Parameters affecting crystal lifetime in MX and possible radiation damage mitigation strategies.

MX Frontiers at the One Micron Scale
BNL, USA.
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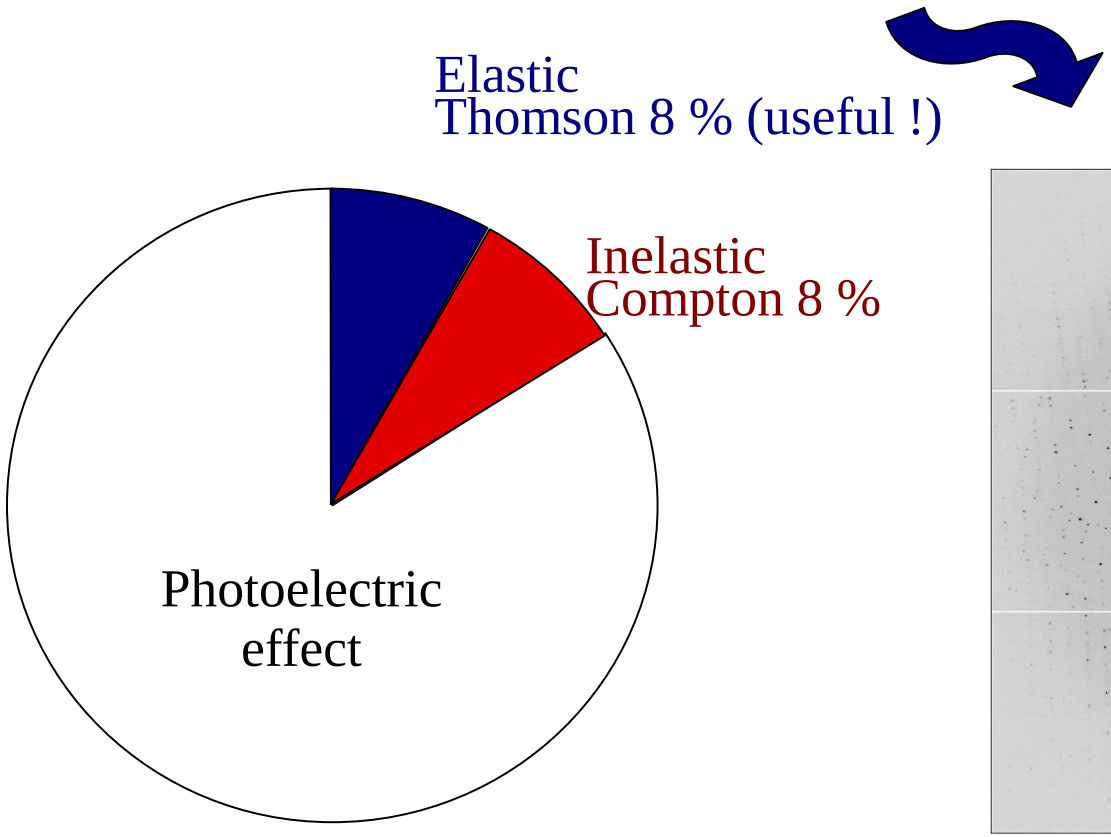
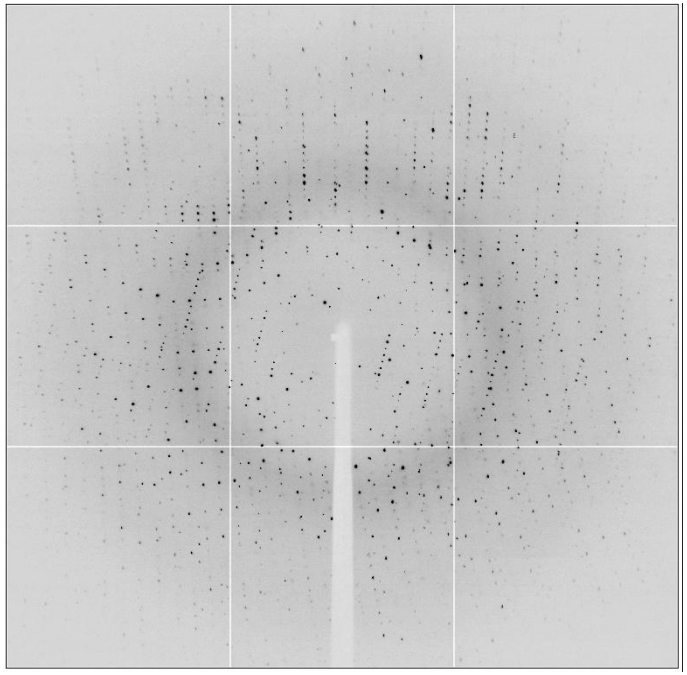
The Plan:

- **A metric for Radiation Damage. Dose: RADDOSE.**
- Scavengers: RT and 100K.
- Simultaneous multi-crystal data collection and data retrieval.

What really happens when X-ray photons hit the crystal ?





λ = 1 Å (at energy 12.4 keV) for a 100x100x100µm crystal

Compton scattering and photoelectric effect both deposit energy in the crystal

DOSE

- DOSE is the ENERGY lost per KILOGRAMME (!!)
- Measured in Joules/kg i.e. the absorbed energy per unit mass.
- Fundamental metric against which to measure damage.
- FLUX is in photons/second.
- Flux density is in photons/second/unit area.
- Takes care of the physics but NOT the chemistry.

DOSE Postulate:

- Damage at 100K is proportional to dose (I_{mean}, Bfactor, R_{merge}, R_d, specific structural damage [dose rate effect?]).
- There is a MAXIMUM dose (Joules/kg = Gy) which protein crystals can tolerate which depends only on the PHYSICS of the situation.
- Crystal might not reach that limit due to chemical factors, but it will not last BEYOND the limit.
- Need to be able to calculate this DOSE:

  [RADDOSE: Murray, Garman & Ravelli, JAPC 2004]

Way of estimating absorbed dose, D: (Gy = J kg^{-1})

Dose rate = mass absorption coeff * photon energy * number of photons in unit time / Area

$$dD/dt = (\mu/\rho)\ E\ I_{inc}$$ (I_{inc}= incident flux density)

For (μ/ρ) in cm^2/g, I_{inc} in photons/s/μm^2, E in keV, t in seconds, total dose is:

$$D = (\mu/\rho)\ E\ I_{inc}\ t\ 10^{11}\ (Gy)$$

e.g. (μ/ρ) = 2.6 cm^2/g (50% solvent), E= 12 keV,

t= 100 μm, A = 80 x 80 μm^2 beam cross section

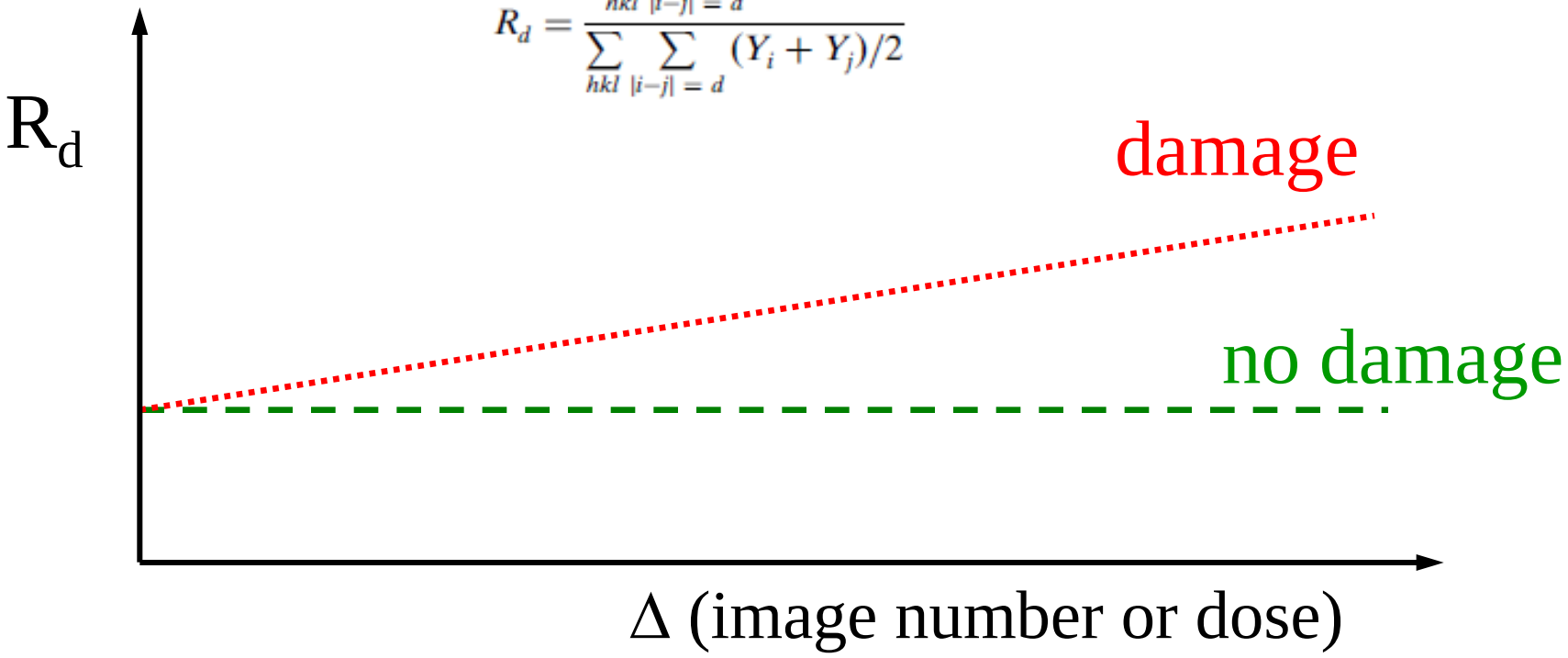
D= 7.8 x 10^{-8} Gy/photon

- For 10^6 Gy, 1 ionisation / 20 amino acids for a 400 a.a. protein molecule. [See O'Neill, Stevens & Garman. JSR (2002) **9**, 329-332]

Can define Decay R_{factor} to plot against Δ(dose):

R_d : pair wise R factor between identical and symmetry related reflections occurring on different diffraction images.

$$R_d = \frac{\sum_{hkl} \sum_{|i-j|=d} |Y_i - Y_j|}{\sum_{hkl} \sum_{|i-j|=d} (Y_i + Y_j)/2}$$

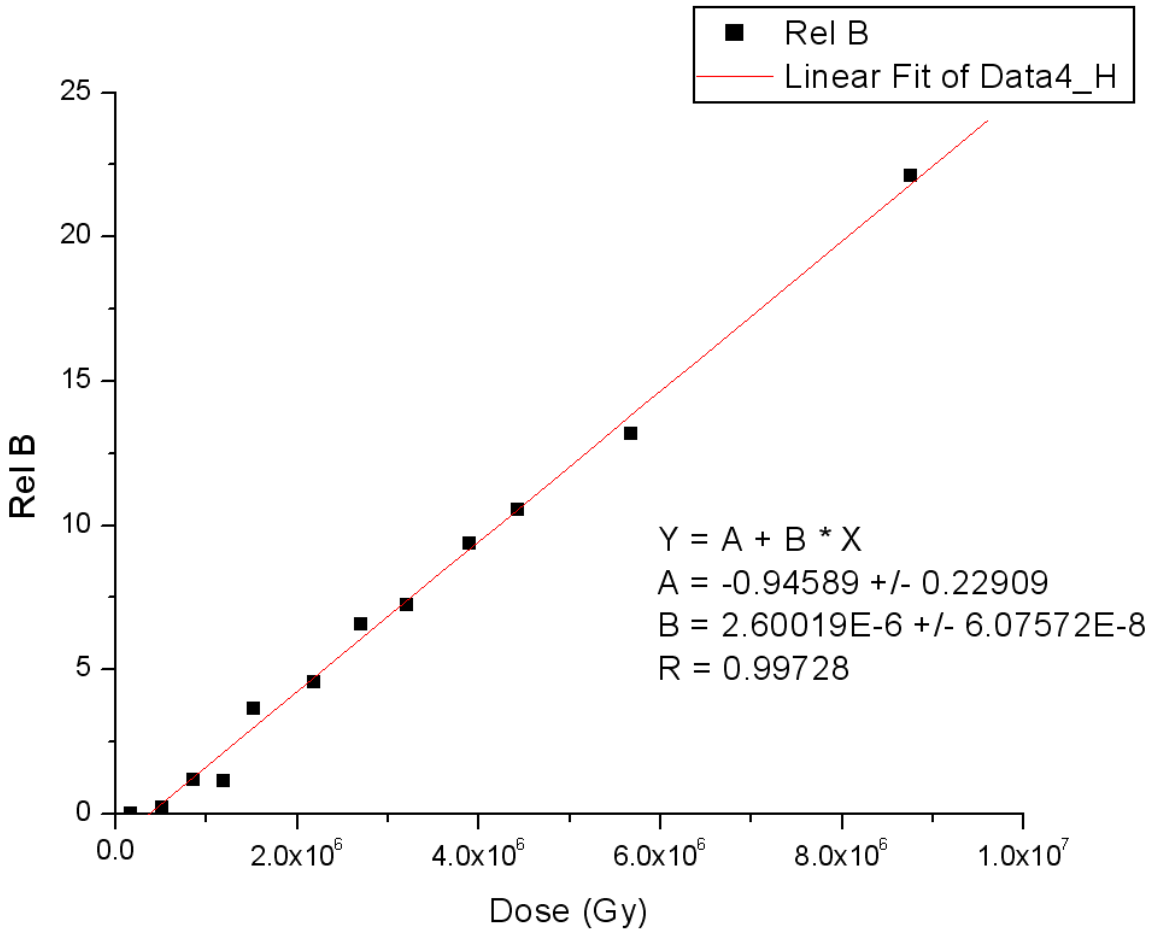


[Deiderichs 2006 Acta D59, 903]

Can define a s_{AD} (to plot against dose):

- Coefficient of sensitivity α change in relative isotropic B factor: [Kmetko et al 2006, Acta D62, 1030]

$s_{AD} = \Delta B_{rel} / 8\pi^2 \Delta D$ (e.g. HEWL= 0.012 at 100K)



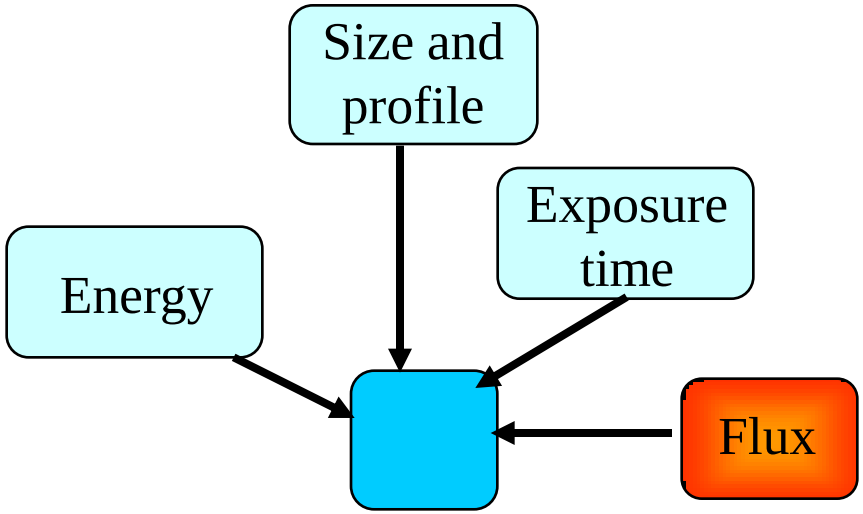
Make this easier for MX (include solvent contribution in mM and heavy atoms explicitly)

RADDOSE

Use crystal and beam characteristics to calculate the dose.

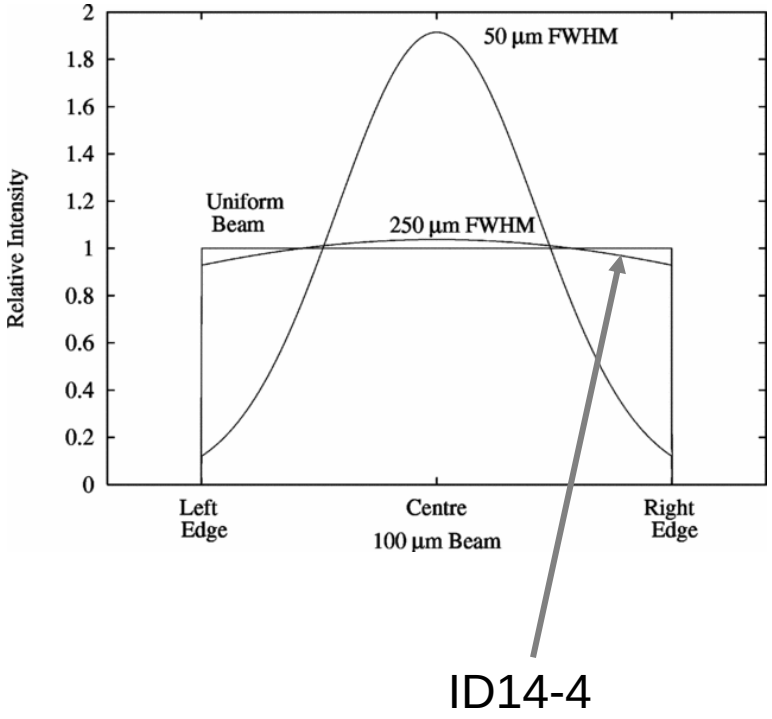
Calculating Dose
(*RADDOSE*)

Beam Characteristics

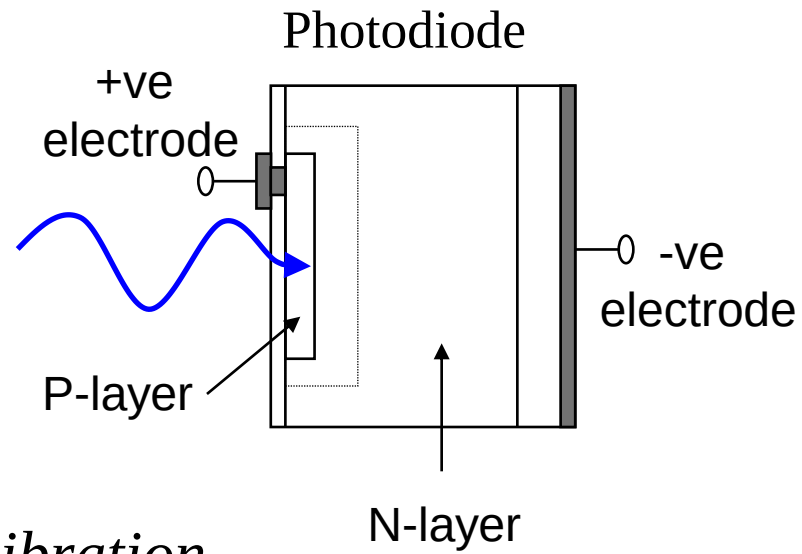


Beam Characteristics

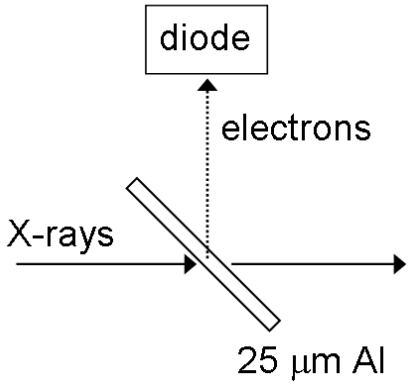
Beam profile



Photons per second

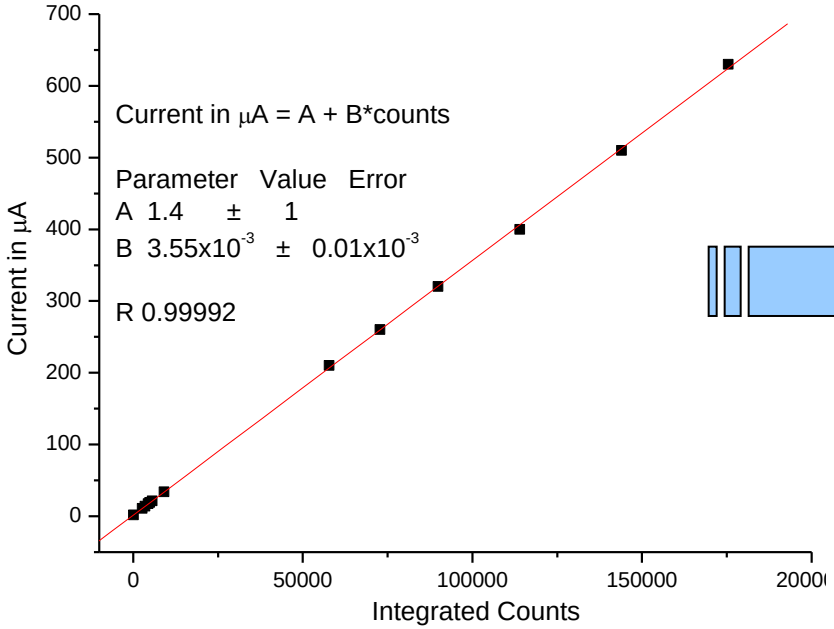


Calibration

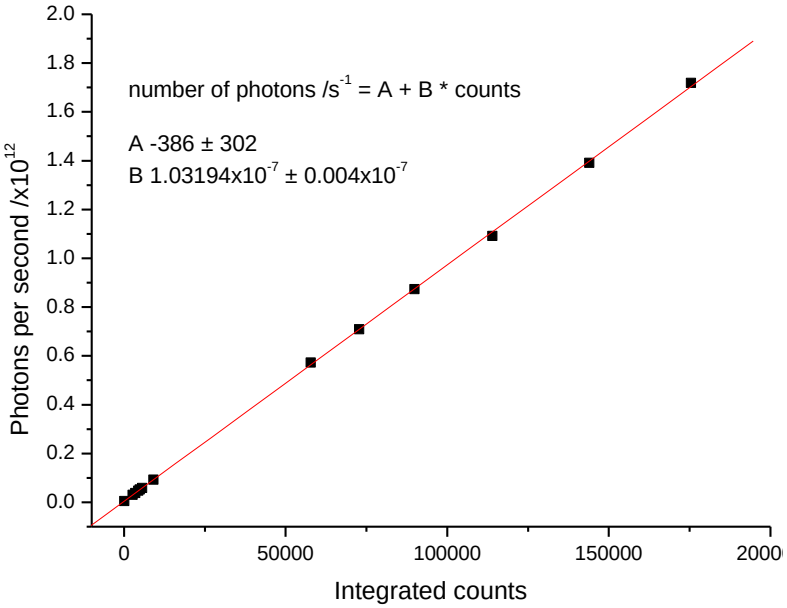


Wavelength

Beam Characteristics



Pin-diode current vs. integrated counts at different attenuations
Hamamatsu Si (S3204-09)

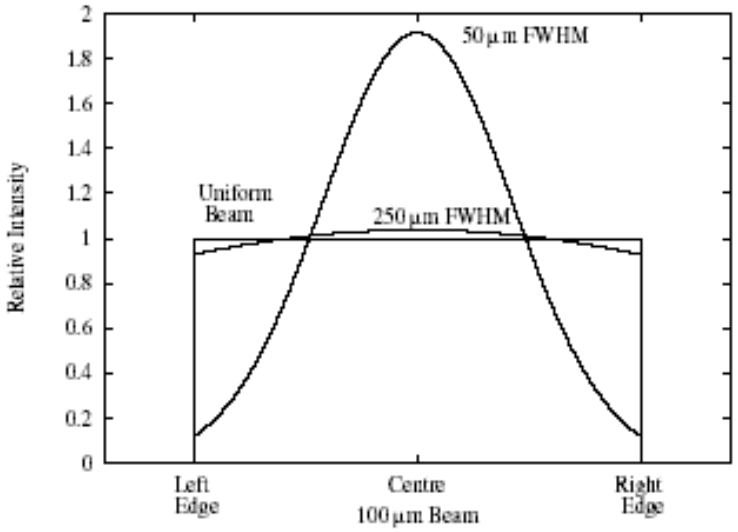


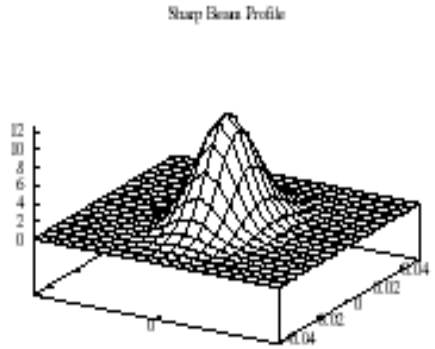
Photons per second vs. integrated counts

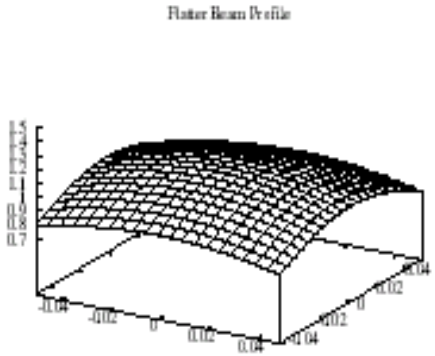
N.B.; Two calibrations required.

Determination of X-ray flux using silicon pin diodes. RL Owen, JM Holton, C Schulze-Briese, EF Garman. *JSR* (2009) **16,** 143-151.

Beam Profile Comparison uniform or 2D Gaussians





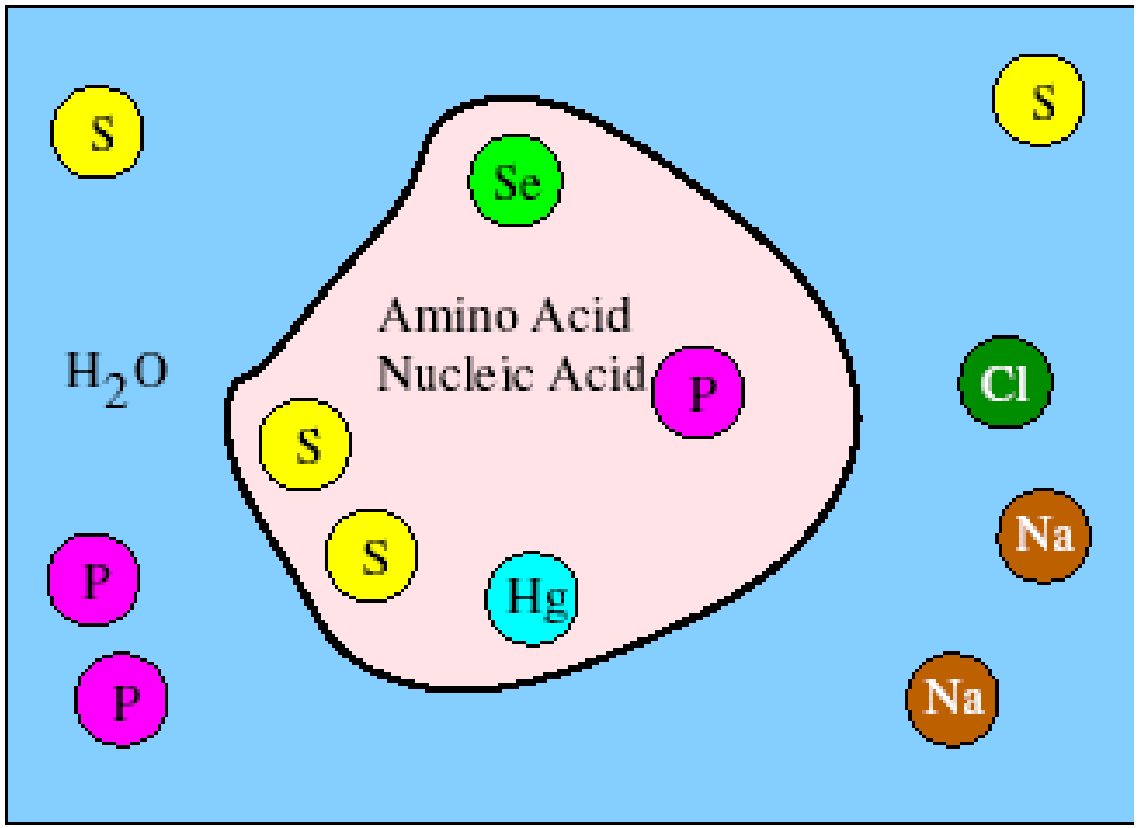


Beam Profiles may be entered into RADDOSE as uniform or 2D Gaussians

- Lifetime curve is reciprocal of intensity curve
- Differential irradiation may lead to differential damage

  get data which merge poorly

- Crystal heating might be higher than first predicted



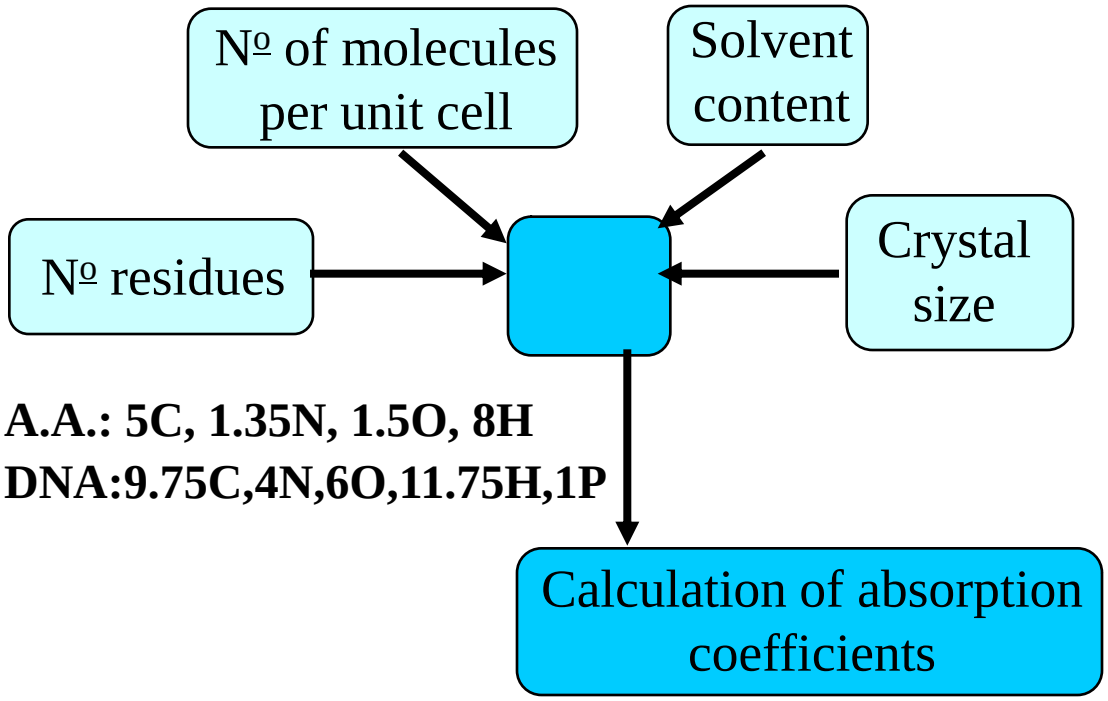
Number of amino acids

'HA' atoms per monomer, *e.g.* S, Se, Hg

Solvent - concentrations of components, *e.g.* Na^+, Cl^-

Calculating Dose
(*RADDOSE*)

Crystal Characteristics



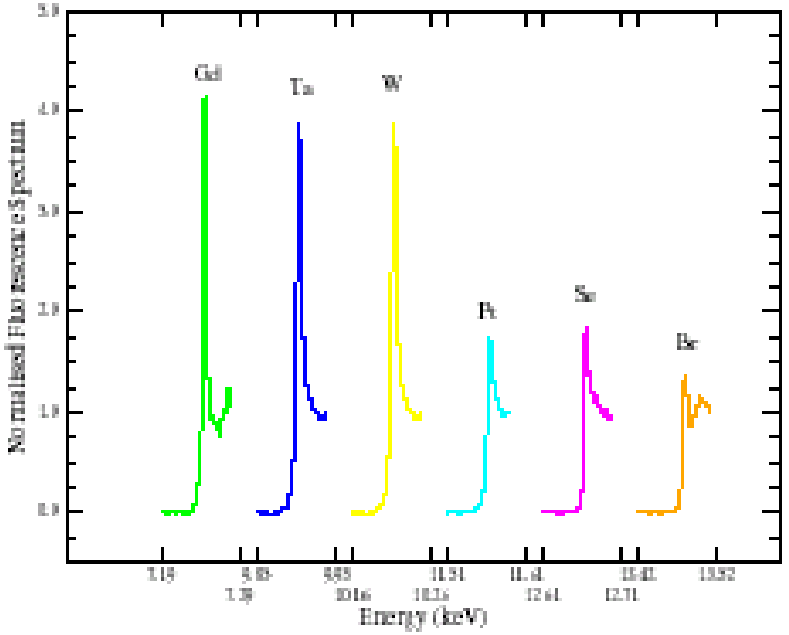
absorption coefficients at 12.4 keV

e.g. apoferritin: 0.406mm^{-1}

holoferritin: 1.133mm^{-1}

Experimental Absorption Coefficients for heavy atoms

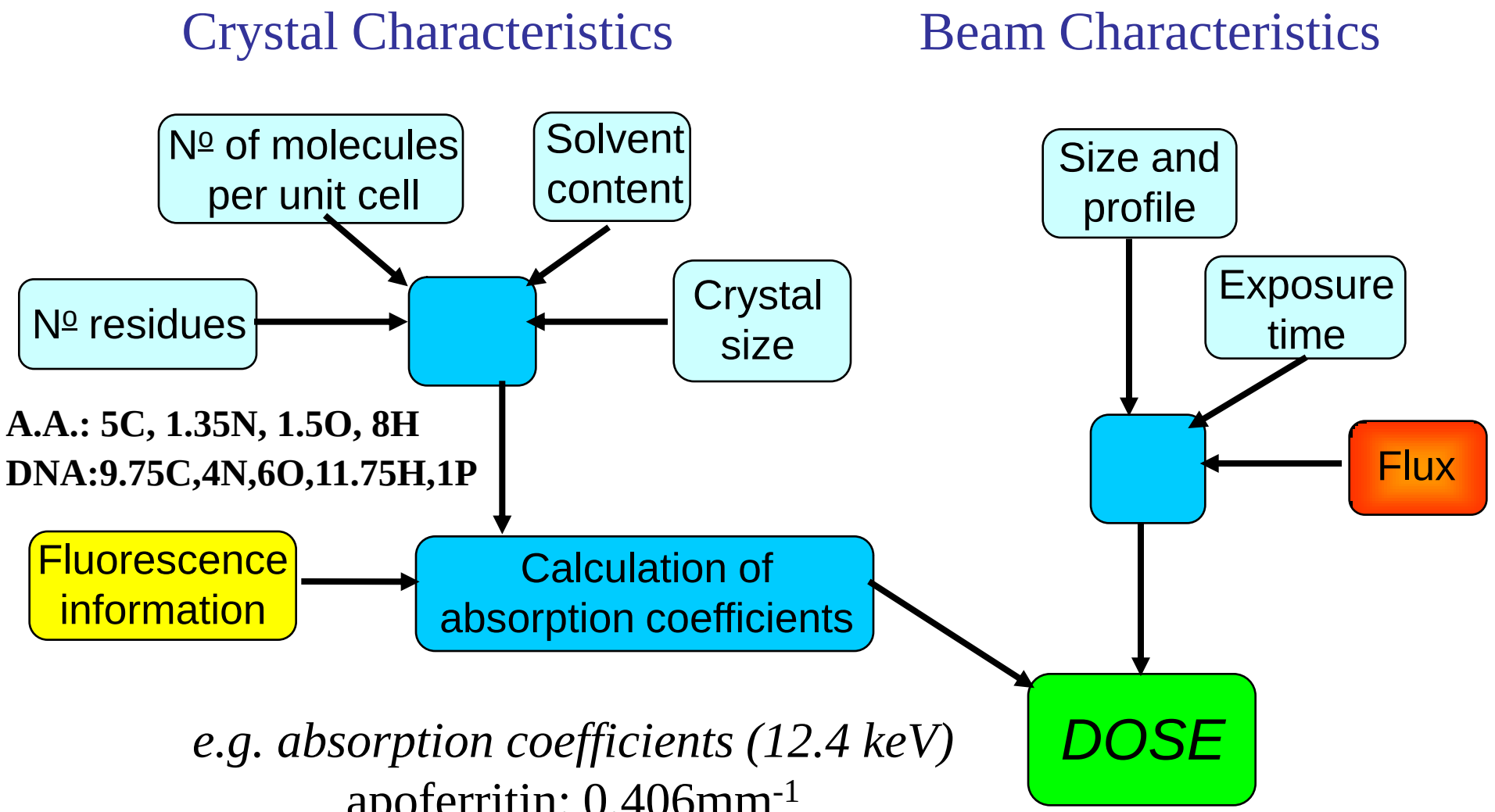
Variety of anomalous edges



f'' is proportional to μ_{pe}

- One can normalise a fluorescence spectrum to known values of
- μ_{pe} far from the absorption edge.
- This is implemented in RADDOSE using the SPLINOR file from CHOOCH. (G. Evans and R. F. Pettifer *J. Appl. Cryst.* **34**, 82-86, 2001.)
- Other absorption values are taken from library values McMaster 1960 and mucal.f

Calculating Dose
(*RADDOSE*)



e.g. absorption coefficients (12.4 keV)

apoferritin: 0.406mm^{-1}

holoferritin: 1.133mm^{-1}

Experimental Dose Limit (100K)

For I_0 1/2

$D_{1/2}$ = 4.3 (± 0.4) 10^7 Gy = 43 MGy

(cf `Henderson limit' 20 MGy ≡ 5 electrons/Å^2
43 MGy ≅ 10 electrons/Å^2
cf. hamster death 3 Gy)

Suggested limit to retain biological `fidelity'

I_0 0.7

$D_{0.7}$ = 3.0 10^7 Gy =**30 MGy**

$D_{0.7}$ for ferritin corresponds 107 photons/unit cell

Robin Leslie Owen, Enrique Rudiño-Piñera, Elspeth F. Garman.
PNAS (2006) 103, 4912 - 4917.

Assumptions in distributed version of RADDOSE

- $\mu_{abs} = \mu_{pe}$ small underestimate at high energies: as Compton scattering is neglected
- Fluorescent X-rays are absorbed:
  results in an overestimate of dose for heavy scatterers
- Crystal rotation neglected
- No potential dose rate effect considered.

`X-ray Absorption by Macromolecular Crystals; the Effects of Wavelength and Crystal Composition on Absorbed Dose'. Murray, Garman, Ravelli, *J. Appl. Cryst.* (2004) 26, 513-522

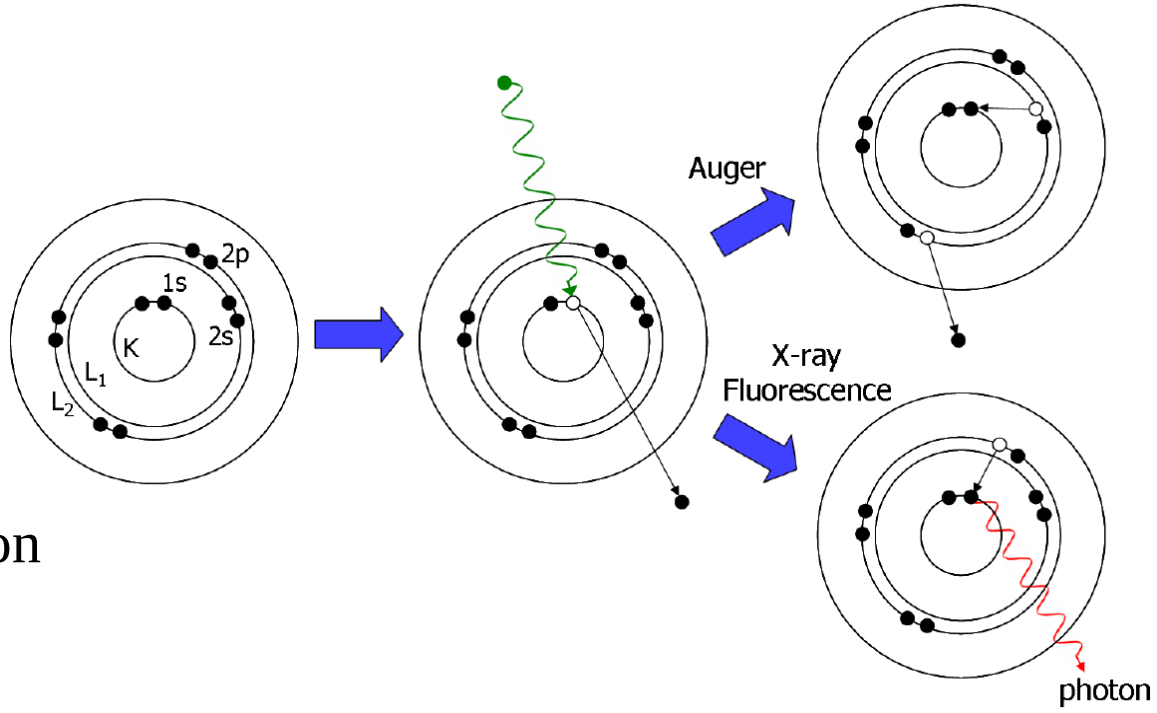
Improved RADDOSE (2009)

- Input/Output made independent of CCP4 libraries
- Outputs time to reach experimental dose limit (30 MGy)
- Makes a correction related to physics of energy loss

For atoms with Z > 20 after interaction via photo electric effect the electron can relax via

1. Auger effect
2. X-ray fluorescence

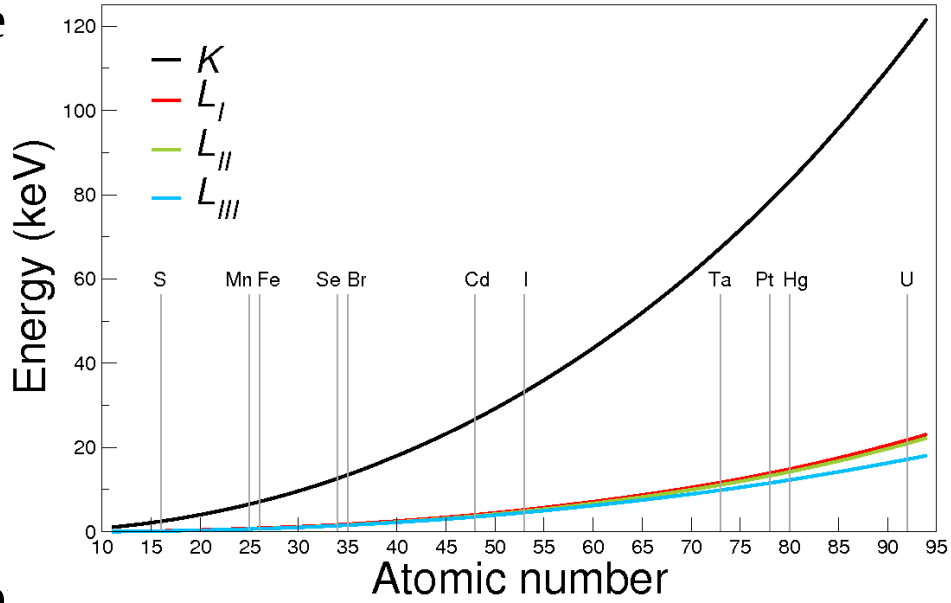
This fluorescence energy may escape from crystal.

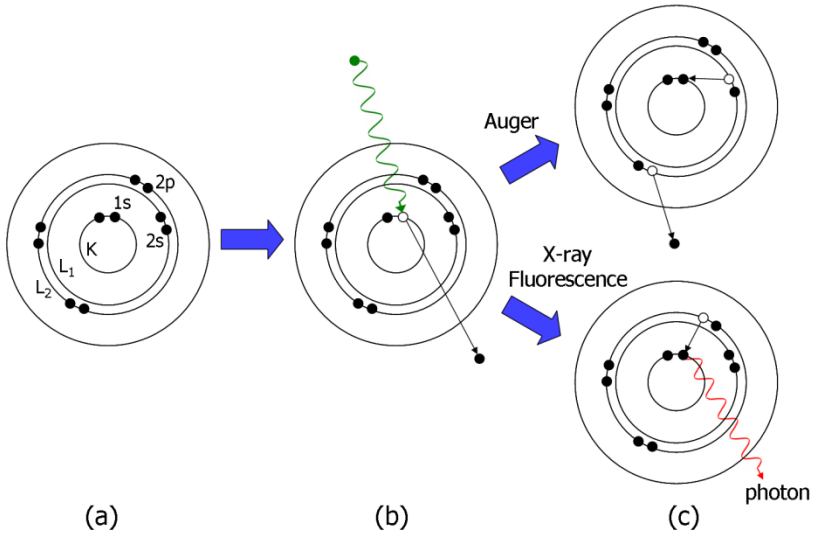


- Energy loss from Compton scattering included.

Why care about X-ray fluorescence escape ?

- If the incident energy is greater than the absorption edge energy; that atom may undergo photoelectric excitation
- Atom can decay *via* Auger or X-ray fluorescence
- X-ray fluorescence photon can escape, depending on its energy, the thickness of crystal, decreasing the energy lost in the sample.
- **May be important for micro crystals**
- Knowledge of correct dose → correct estimation of lifetime → planning of the experiment





X-ray fluorescence escape:

- For C, N, O, S there is low probability of X-ray fluorescence.

- For heavy elements (Z > 20) such as Se there is higher chance of X-ray fluorescence

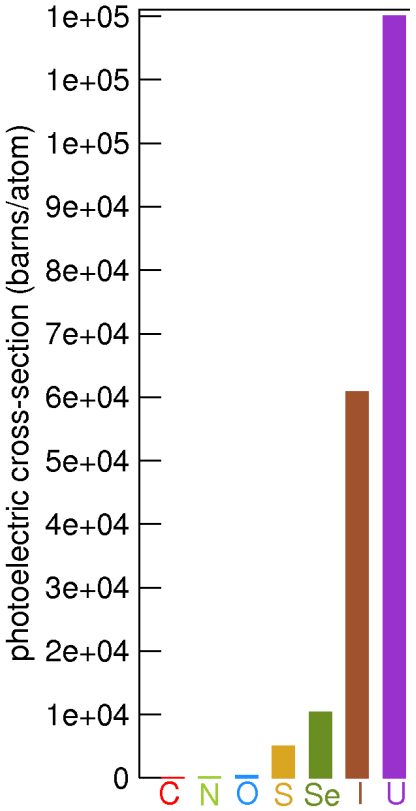
- The contribution to the anomalous scattering factor (f ") is directly proportional to the photoelectric cross-section.

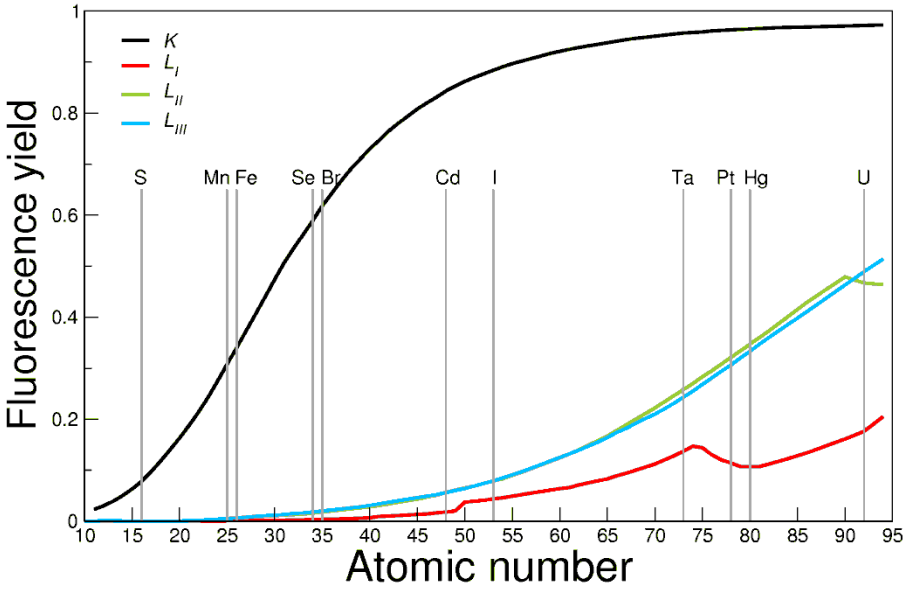
- Corrected energy

  = deposited energy

  – *K*-shell escape, – L_I escape,

  – L_{II} escape, – L_{III} escape



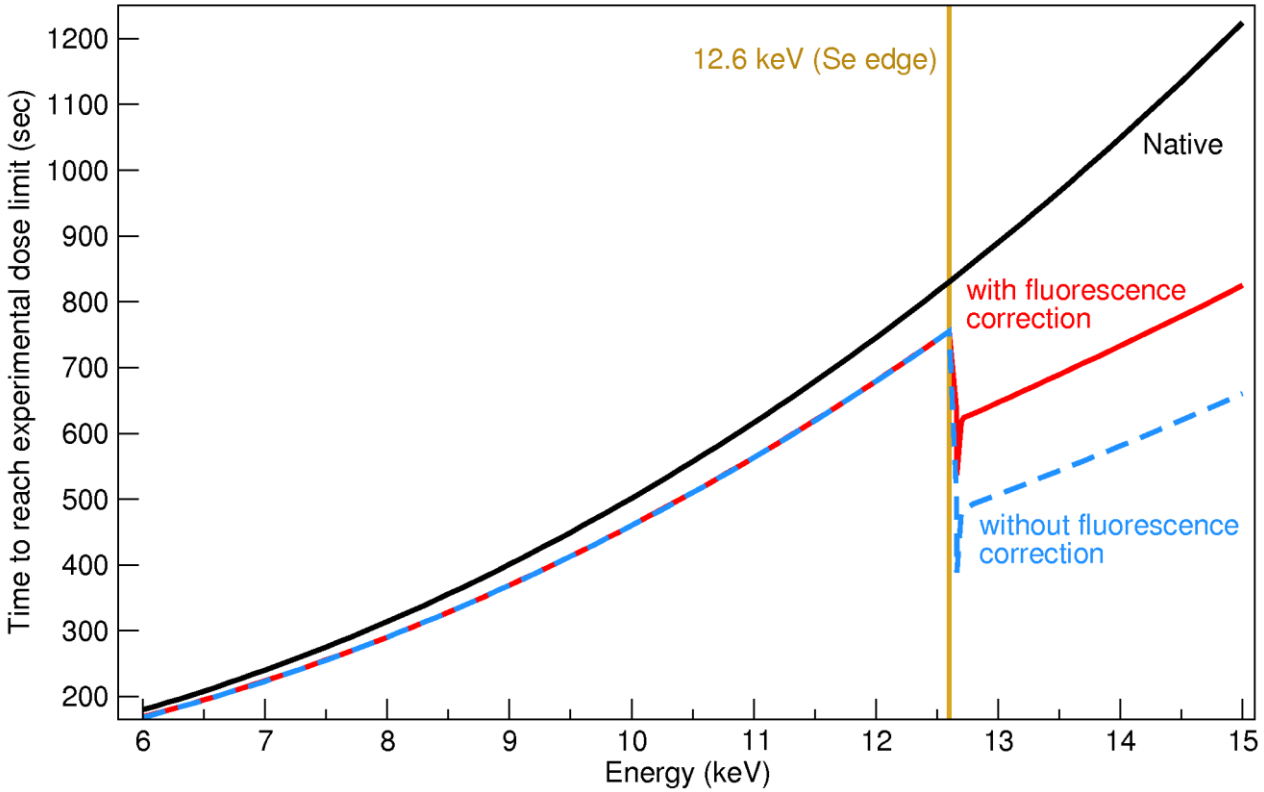


e.g. SeMet protein – phosphomethylpyrimidine kinase

- Seleno-methionine crystals often have a shorter lifetime than native crystal, due to the large photoelectric cross section of selenium.
- Using RADDOSE can predict the maximum crystal lifetime for a MAD experiment: predicted lifetime increases by 27 % at 12.6634 keV when the fluorescent escape is included.

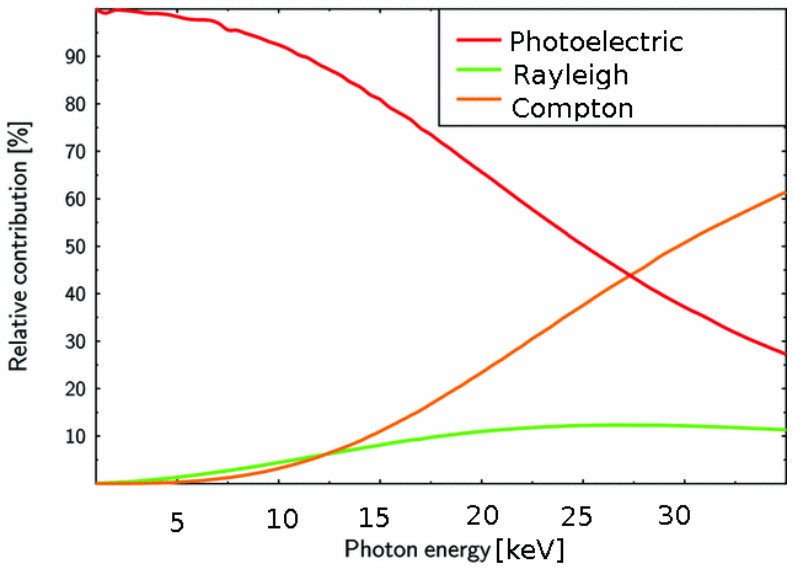
Crystal size:
0.04 0.1 0.05 mm^3

Tophat beam:
0.1 0.1 mm^2



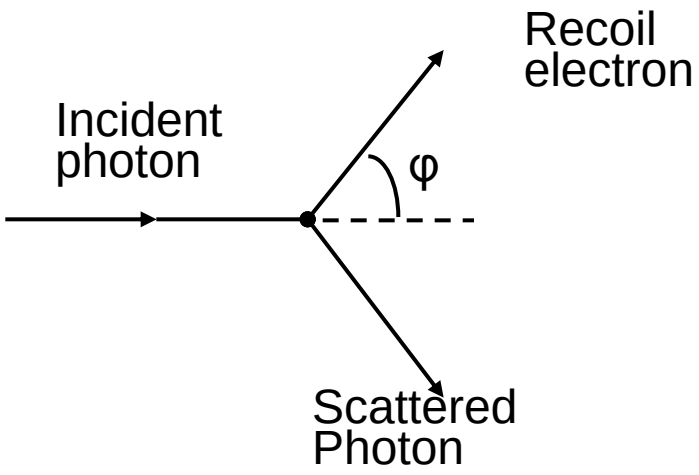
Inclusion of energy absorption due to Compton scattering

- Compton scattering: interaction of a photon with a `free' electron (outer shell)
- The incoming photon will inelastically scatter and the electron will recoil at an angle φ
- The energy of the recoil electron is deposited in the crystal itself (contributes to the absorbed dose)
- Total energy loss $\alpha\ \sigma_{\text{Compton cross-section}}\ E_{\text{recoil electron}} + \sigma_{\text{photoelectric cross-section}}\ E_{\text{photoelectric absorption}}$

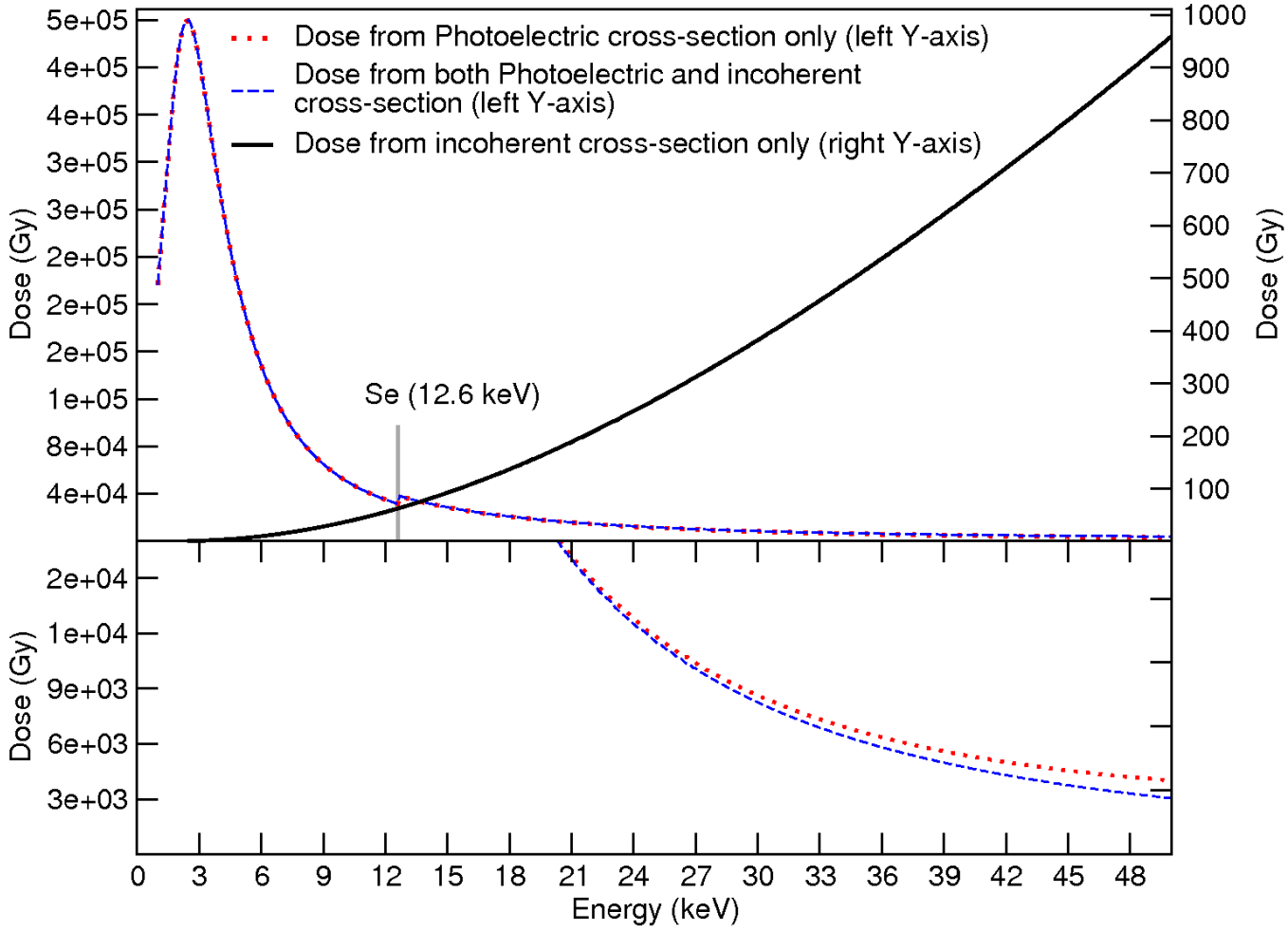


Maximum electron recoil energy:

$$E_{recoiled\ electron} = \frac{2E^2_{incident}}{mc^2\left(1+2\frac{E_{incident}}{mc^2}\right)}$$



Effect of Compton scattering energy loss on dose



PPK, 0.04 0.1 0.05 mm^3 crystal, tophat beam, 0.05 0.05 mm^2, 10^{12} photons/mm^2, 0.2 s/image. 398 residues. 12 seleniums

Diffraction-dose efficiency

- Want to maximise:

$$\frac{\text{Diffracted intensity}}{\text{Dose}} = I_{DE}$$

$$\frac{I_{scatt}}{\text{Dose}} = \frac{I_{scatt} \times V \times \rho}{\text{Energy absorbed}} \quad \alpha \quad \frac{\lambda^2 \; e^{-\mu t} \times V^2 \times \rho}{\text{Energy absorbed}}$$

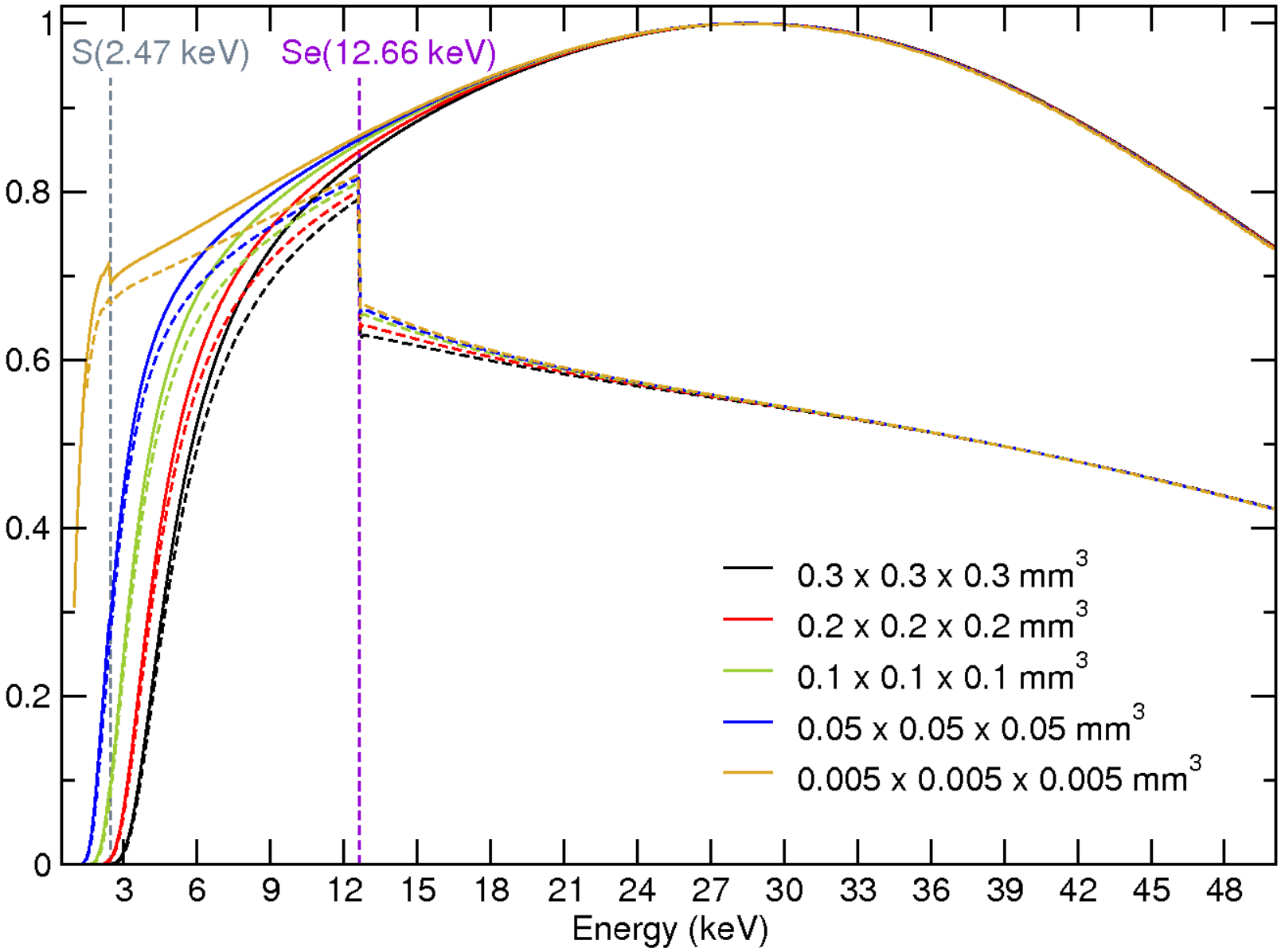
N.B.

[Dose=Energy absorbed/mass]

$$\frac{I_{scatt}}{dose} \alpha \frac{Volume^2 \times \lambda^2 \times e^{-\mu_{att} thickness}}{en_{incident}(1-e^{-\mu_{photoelectric} thickness}) + en_{Comptonelectron}(1-e^{-\mu_{Compton} thickness})}$$

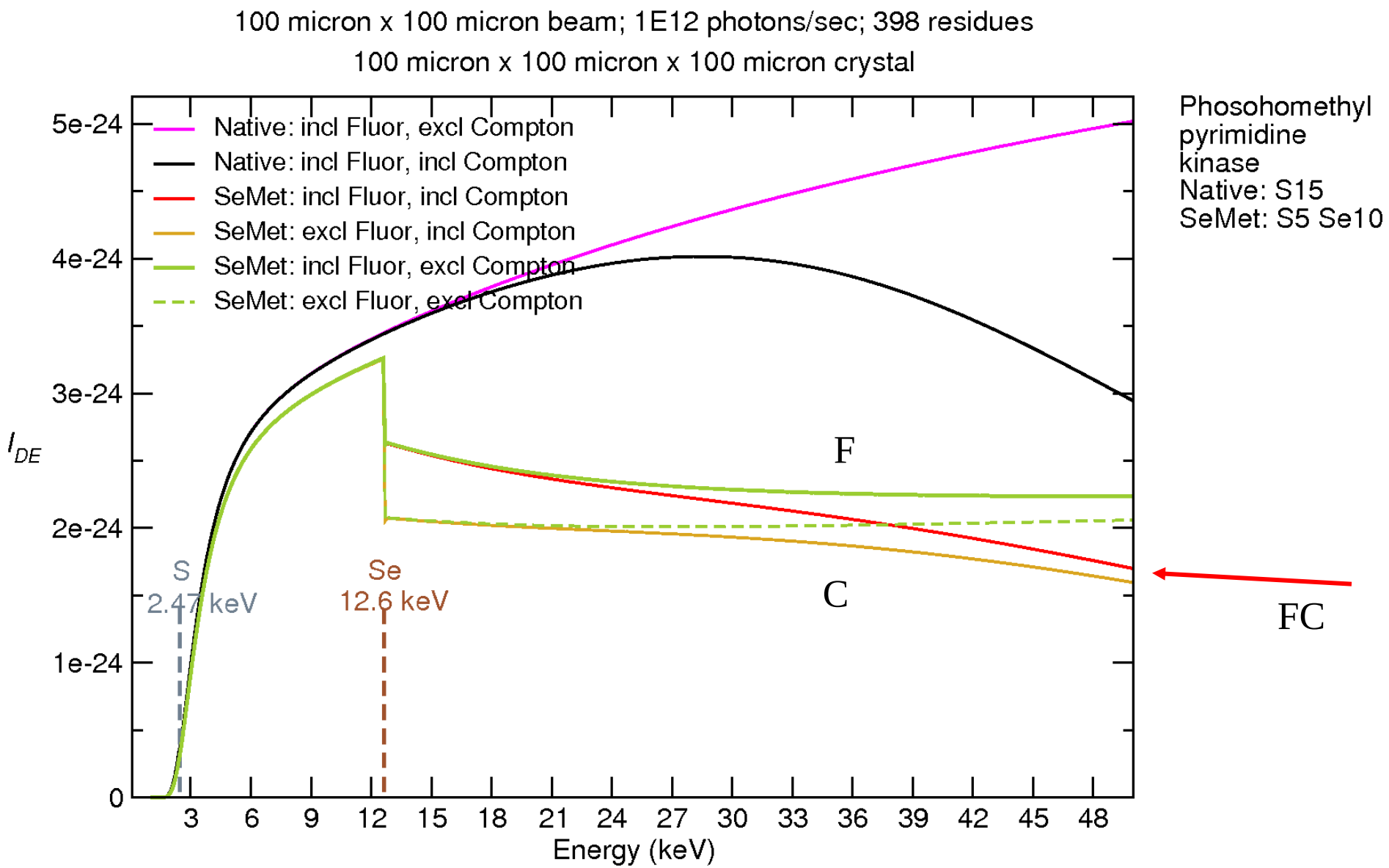
I_{DE}: Is there an optimal energy for a given sized sample ?

Protein:
Phosphomethyl pyrimidine kinase P1
(398 residues)
5 sulphur atoms
12 seleniums
Beam: tophat
10^{12} photons/sec
x,*y* = 0.3 x 0.3 mm^2

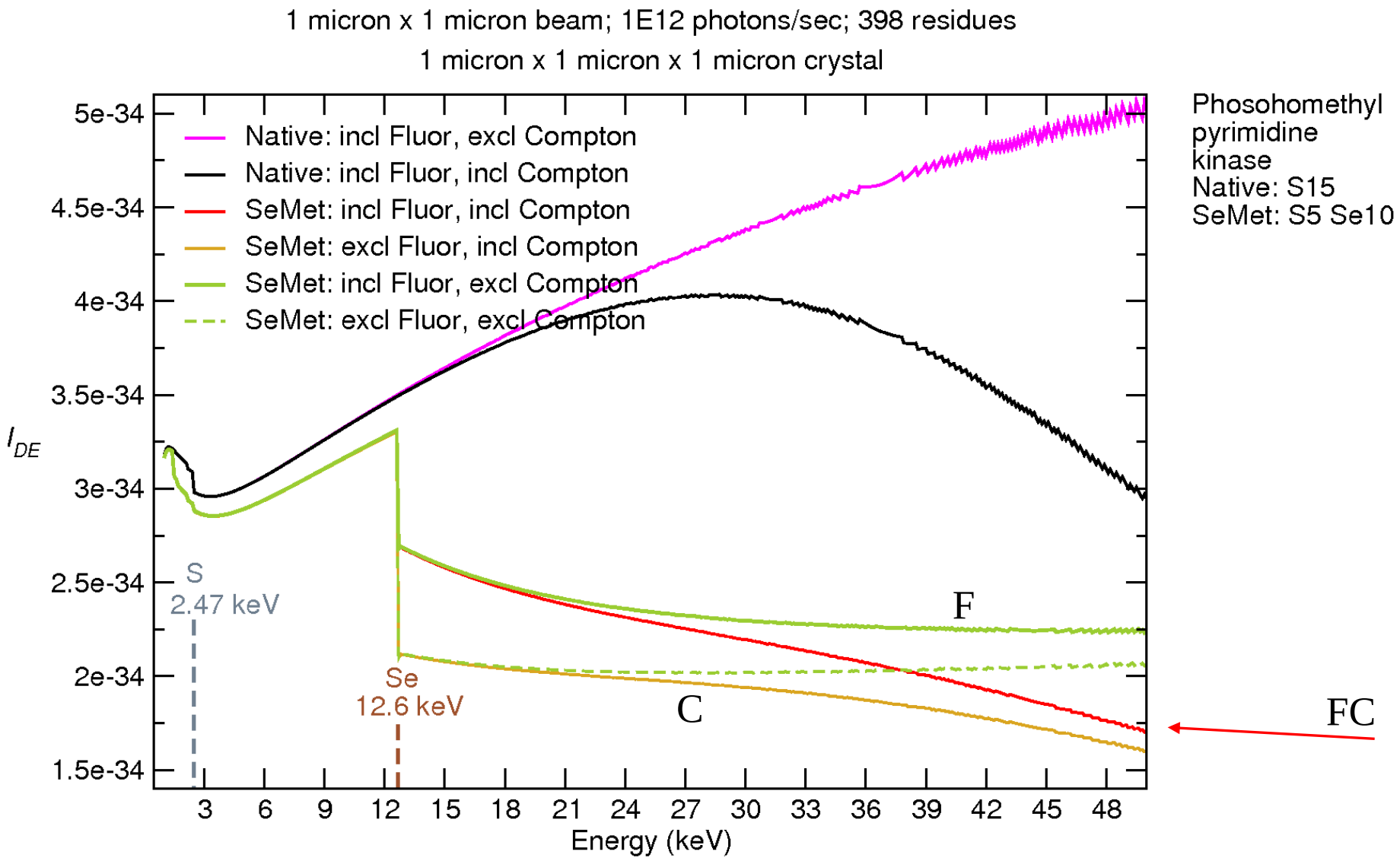


Paithankar & Garman, *in preparation*

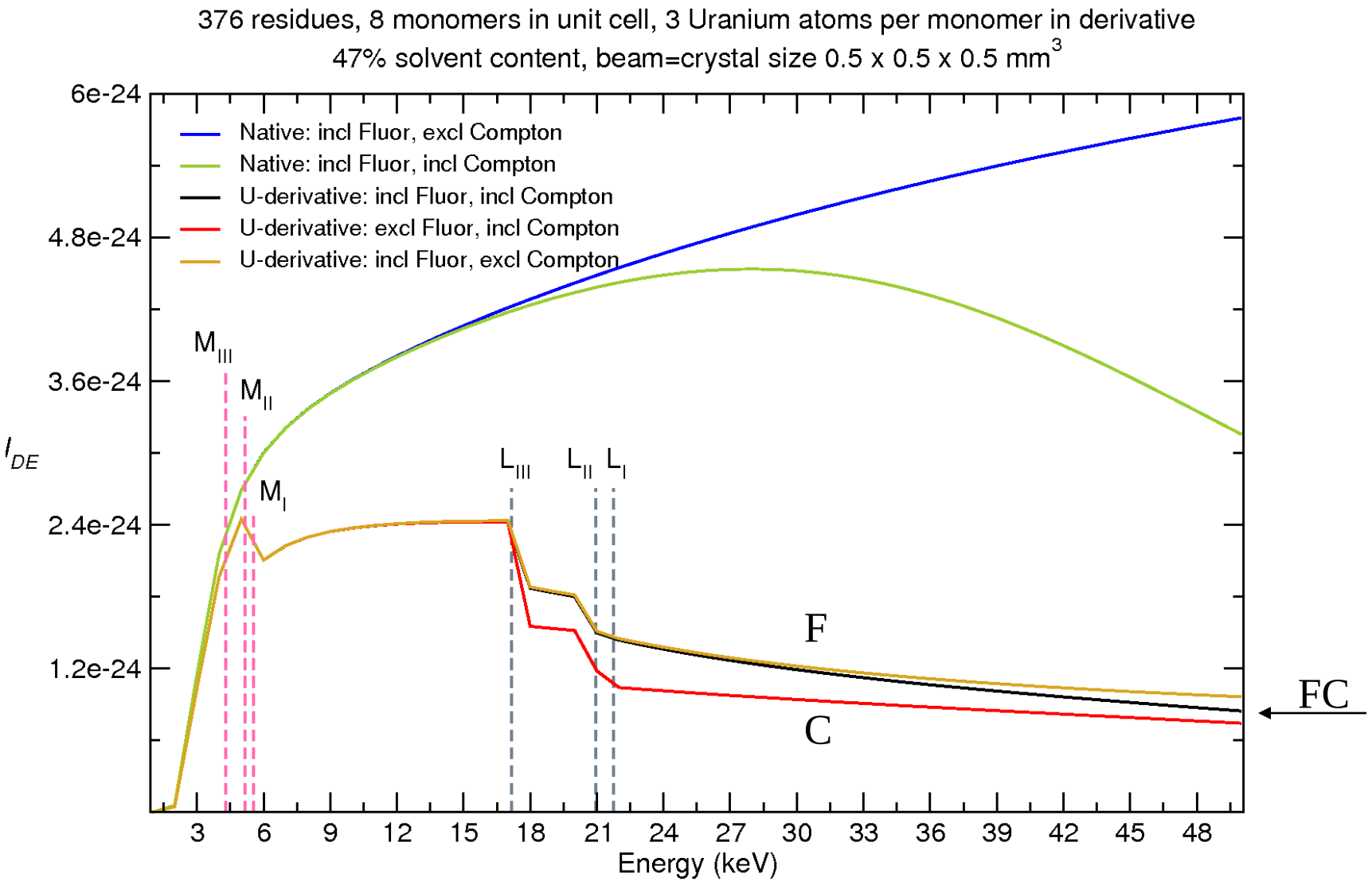
I_{DE} : effect of selenium for a 100µm thick crystal



IDE: effect of selenium for a 1 μm crystal

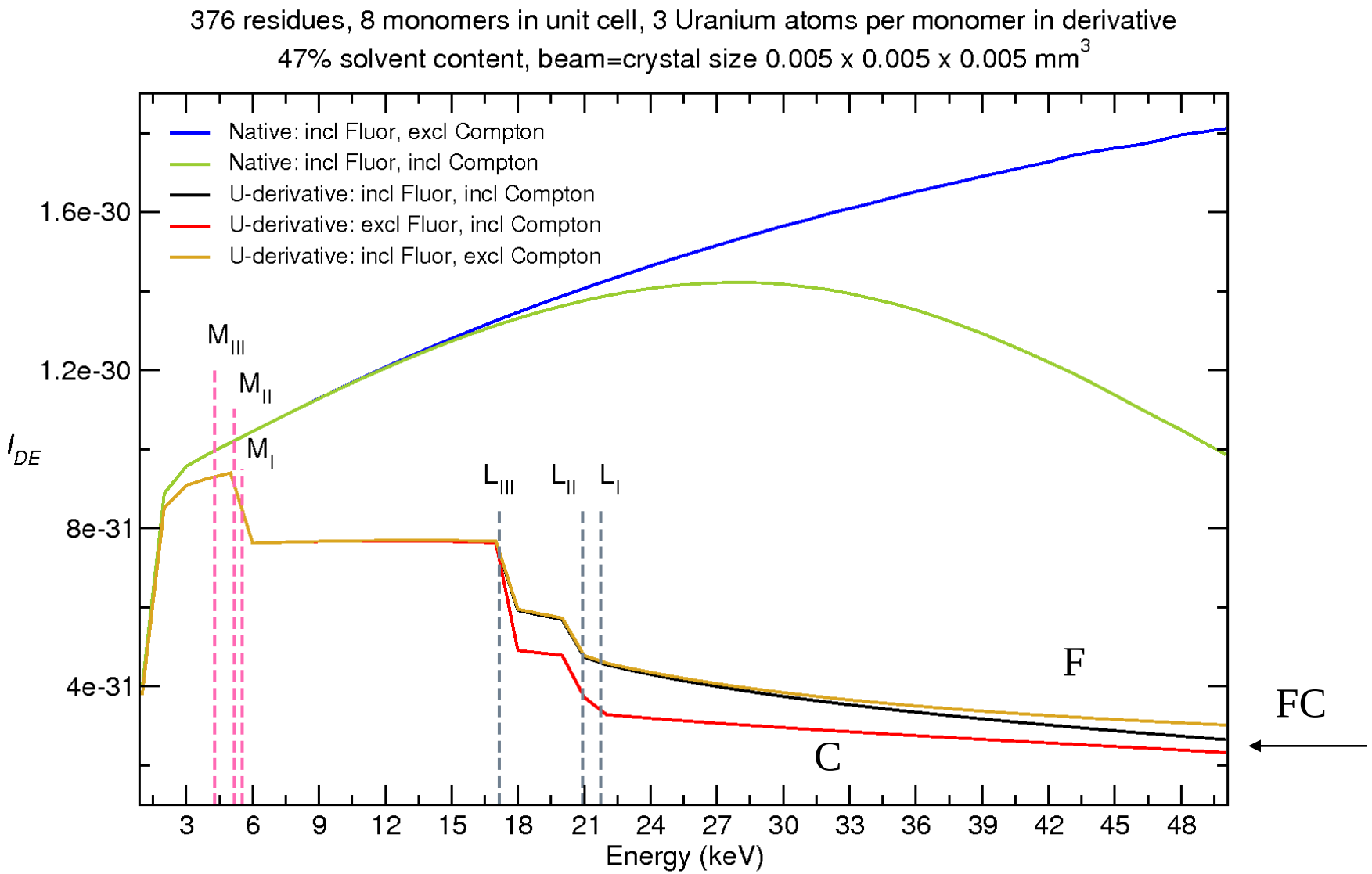


I_{DE}: effect of heavy atoms: 500 μm crystal



human phosphatase binding protein (HPBP)

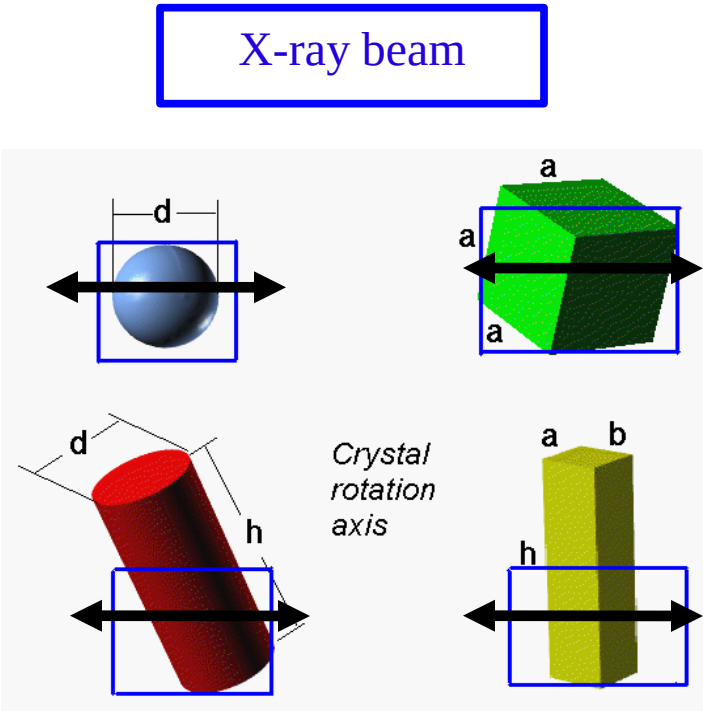
I_{DE} for a 5 μm crystal.



human phosphatase binding protein (HPBP)

Crystal and beam size corrections

- Assume blue box is the X-ray beam perpendicular to the screen and black arrow is the rotation axis
- For crystals whose dimensions exceed that of the beam, dose as calculated for a stationary crystal is not an accurate metric for the estimation of radiation damage
- Taking into account the irradiated volume of the crystal if the crystal is bigger than the beam
- Knowledge of physical orientation of the crystal with respect to X-ray beam

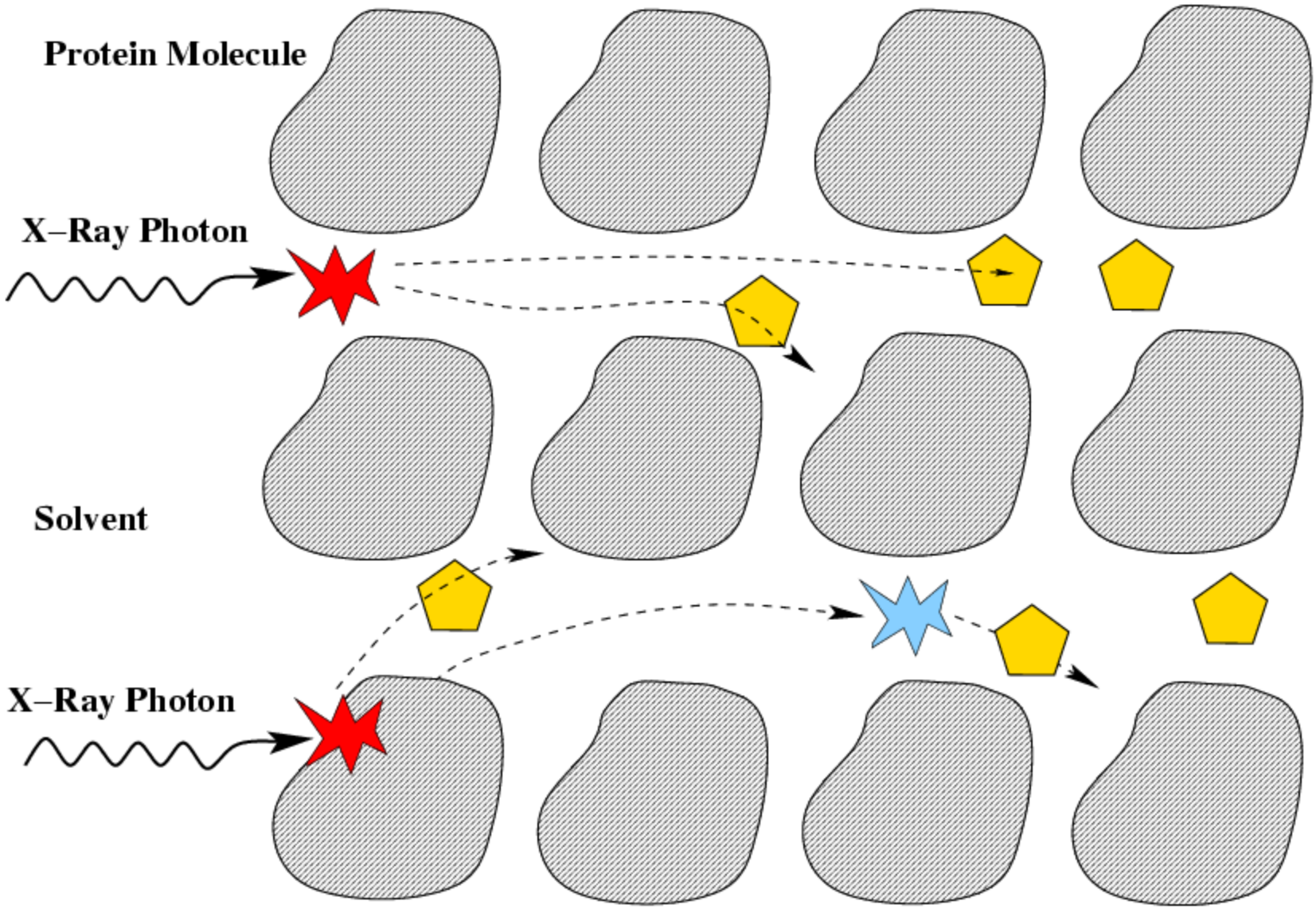


The Plan:

- A metric for Radiation Damage. Dose: RADDOSE.

- **Scavengers: RT and 100K.**

- Simultaneous multi-crystal data collection and data retrieval.


Protein Molecule
X–Ray Photon
Solvent
X–Ray Photon

The chemistry:
mobile e^- go to electron affinic sites

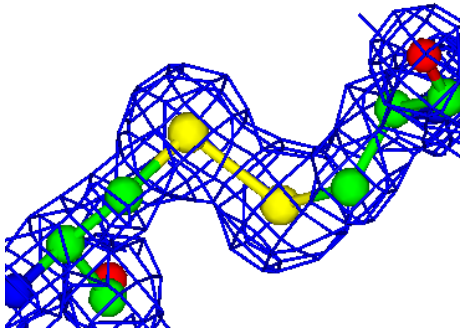
Electron capture $RSSR + e^- \longrightarrow [RSSR]^{\bullet -}$ (400 nm peak)

Disproportionation $[RSSR]^{\bullet -} \longrightarrow RS^- + RS^\bullet$

Protonation $[RSSR]^{\bullet -} + H^+ \longrightarrow RSH + RS^\bullet$

Electron loss $RSSR \longrightarrow [RSSR]^{\bullet +} + e^-$

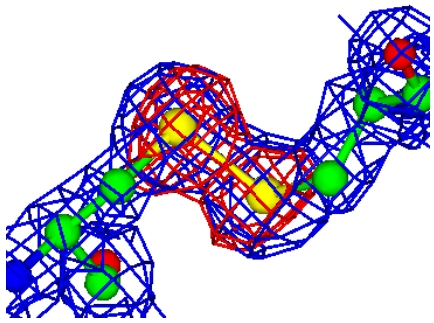
Alkyl loss $RSSR + e^- \longrightarrow RSS^- + R^\bullet$



Specific structural damage

DISULPHIDE BONDS (S-S) MOST SUSCEPTIBLE

Weik *et al* (2000) PNAS 97, 623-628
Burmeister (2000), Acta Cryst D56, 328-341.
Ravelli and McSweeney, (2000) Structure 8, 315-328



Water/solvent chemistry

1) Ionization

Ionizing radiation

$H_2O \rightarrow H_2O^{+\bullet} + e^-$

2) Electronic excitation

Ionizing radiation

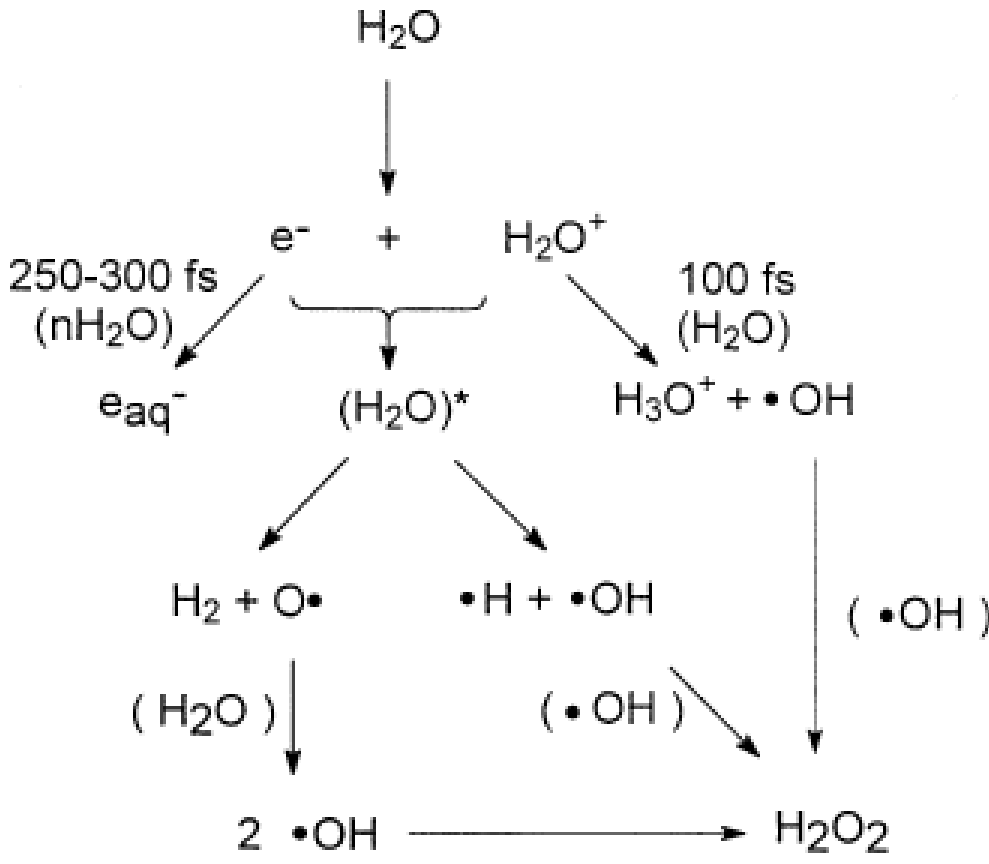
$H_2O \rightarrow H_2O^{+-}$

$H_2O^{+\bullet} + H_2O \rightarrow H_3O^+ + \bullet OH$

$e^- + n\,H_2O \rightarrow e\text{-}aq$

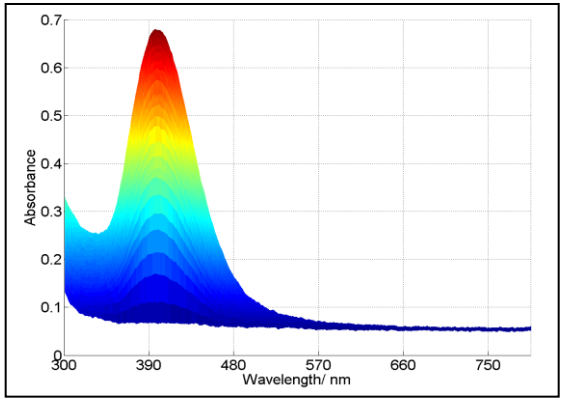
$H_2O^+ \rightarrow H\bullet + \bullet OH$

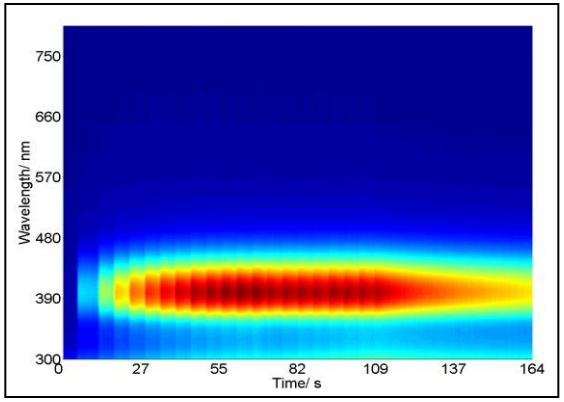
$e\text{-}aq + H+ \rightarrow H\bullet$

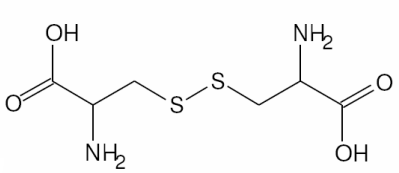


Scavengers at cryotemperatures: Rationale

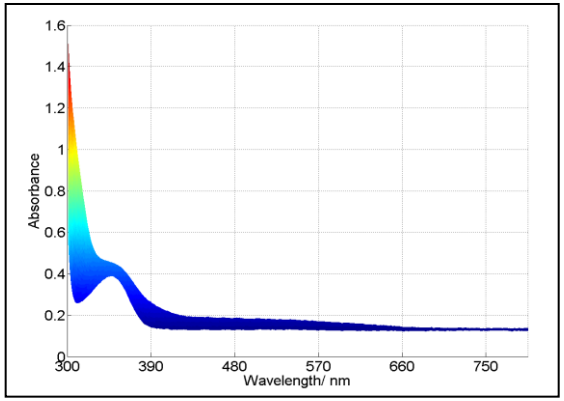
- Crystals are usually cooled to 100K to reduce the mobility of free radicals.
- The existence of specific damage at this temperature shows that some species are still mobile (electrons).
- Therefore scavengers may be able to react with these species and reduce their mobility and the reactivity of fixed species, protecting the crystal from specific damage.

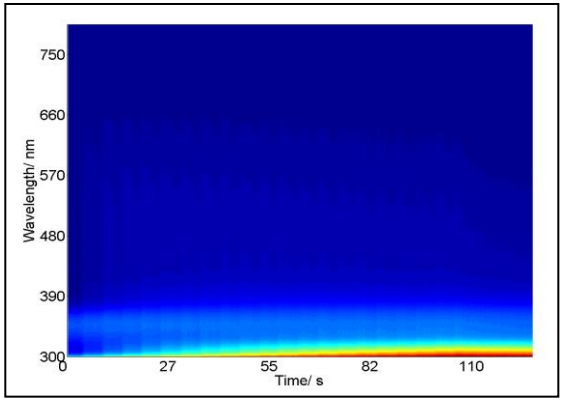


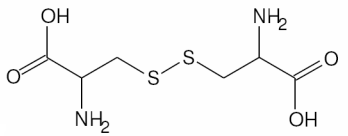




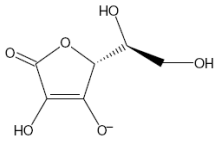
Cystine



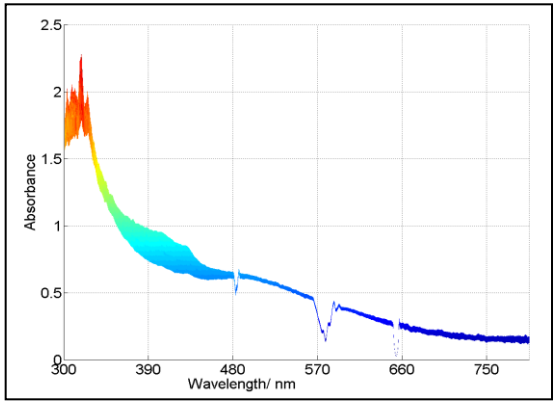


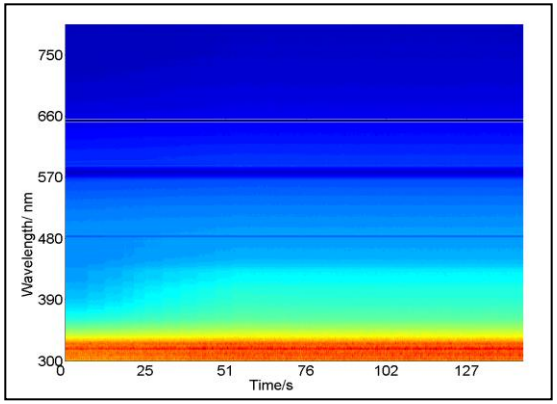


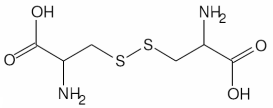
Cystine



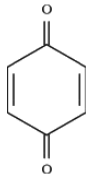
Ascorbate



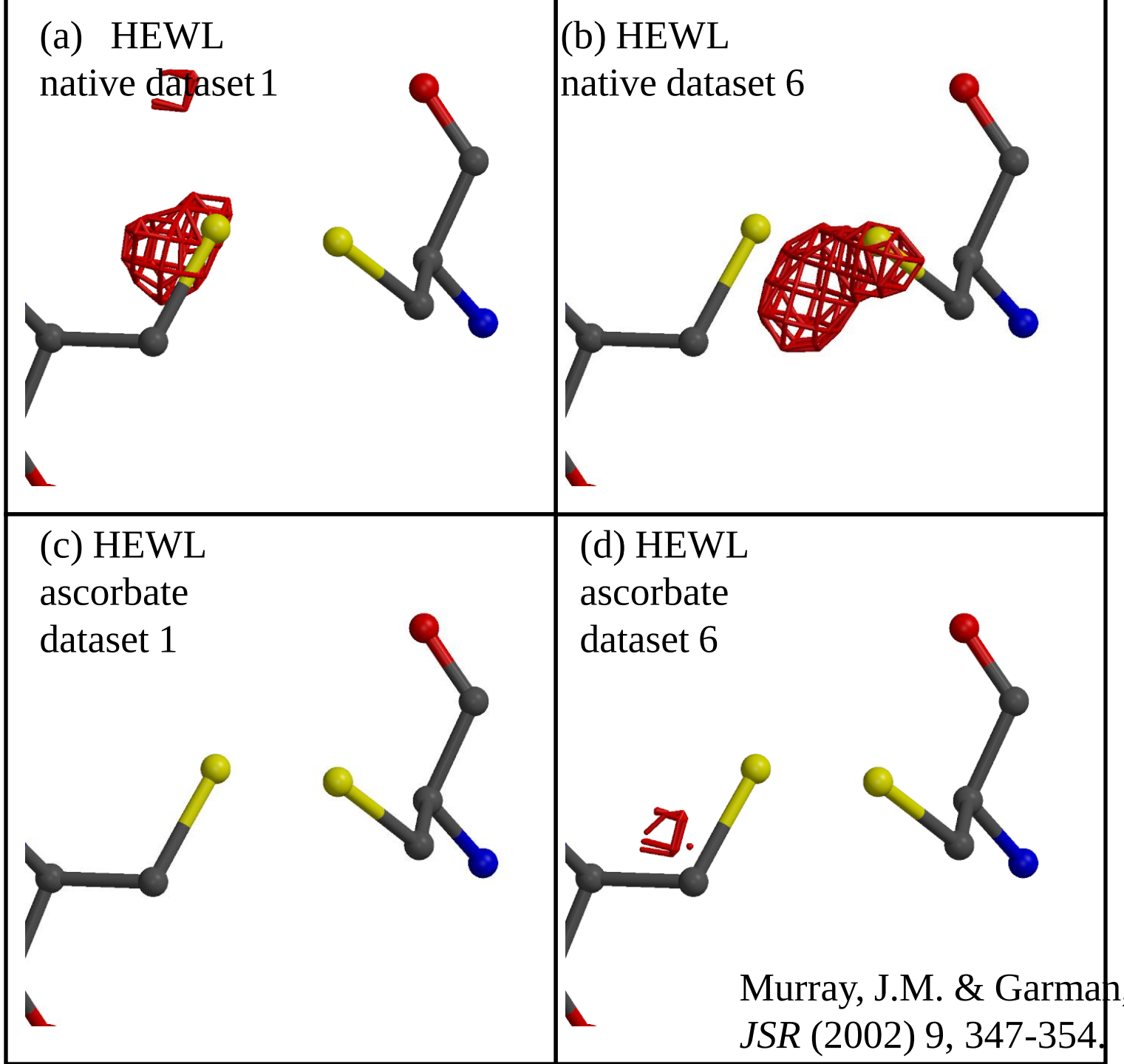




Cystine



Quinone

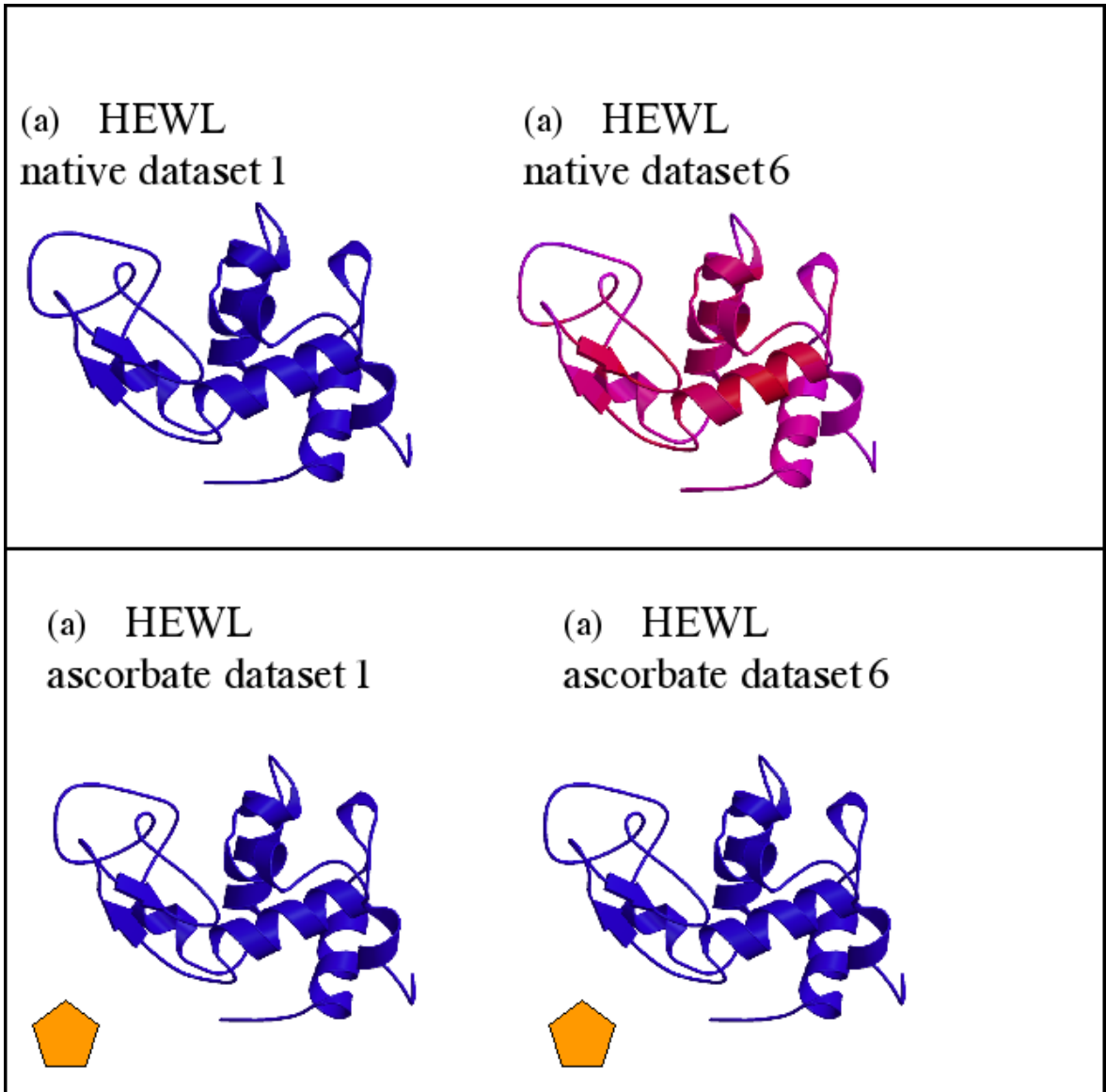


Murray, J.M. & Garman, E.F.
JSR (2002) 9, 347-354.

Change in atomic B factors of refined structures with dose.

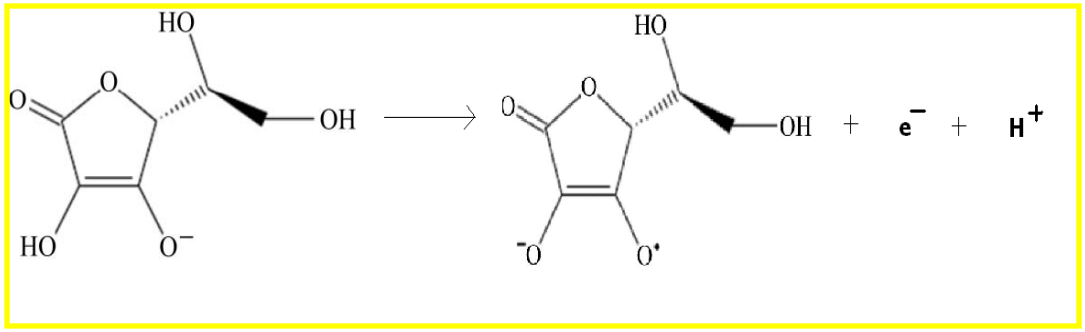
No increase in temperature factor

70 % increase in temperature factor

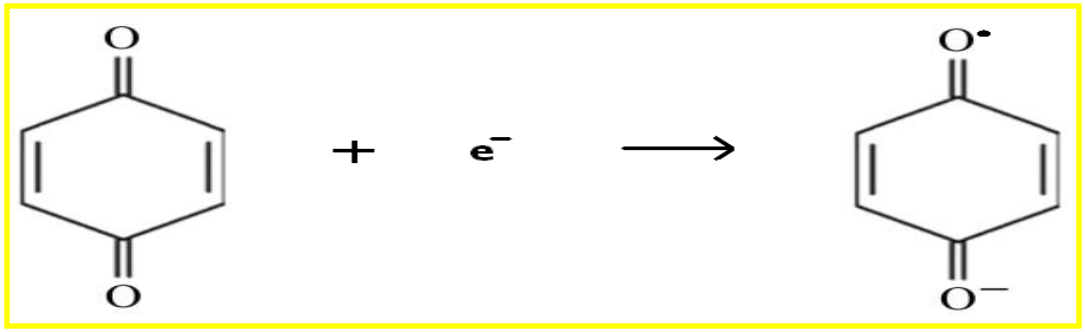


RBG Ravelli & EF Garman *Current Opinion of Structural Biology* (2006) 16, 624-629.

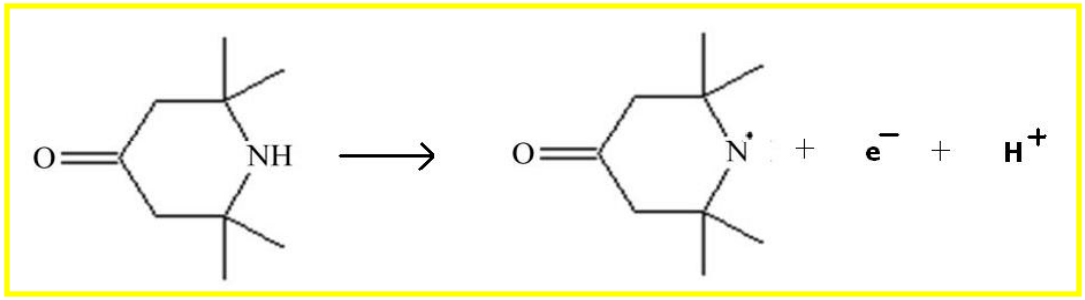
Potential radioprotectants identified by on-line microspectrophotometry



Ascorbate

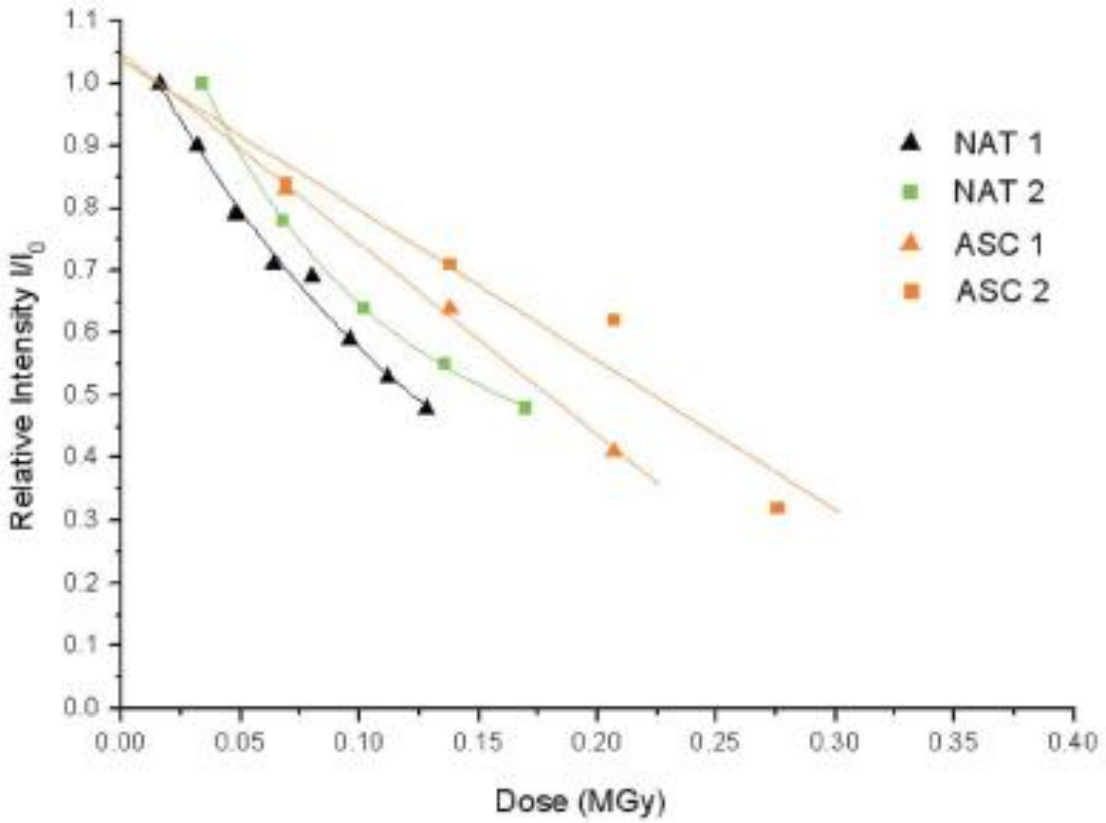


1,4-Benzoquinone



2,2,6,6-tetramethyl-4 -piperidone (TEMP).

RT: Ascorbate, co-crystallised 1M



$D_{1/2}$ Native = 0.125 MGy

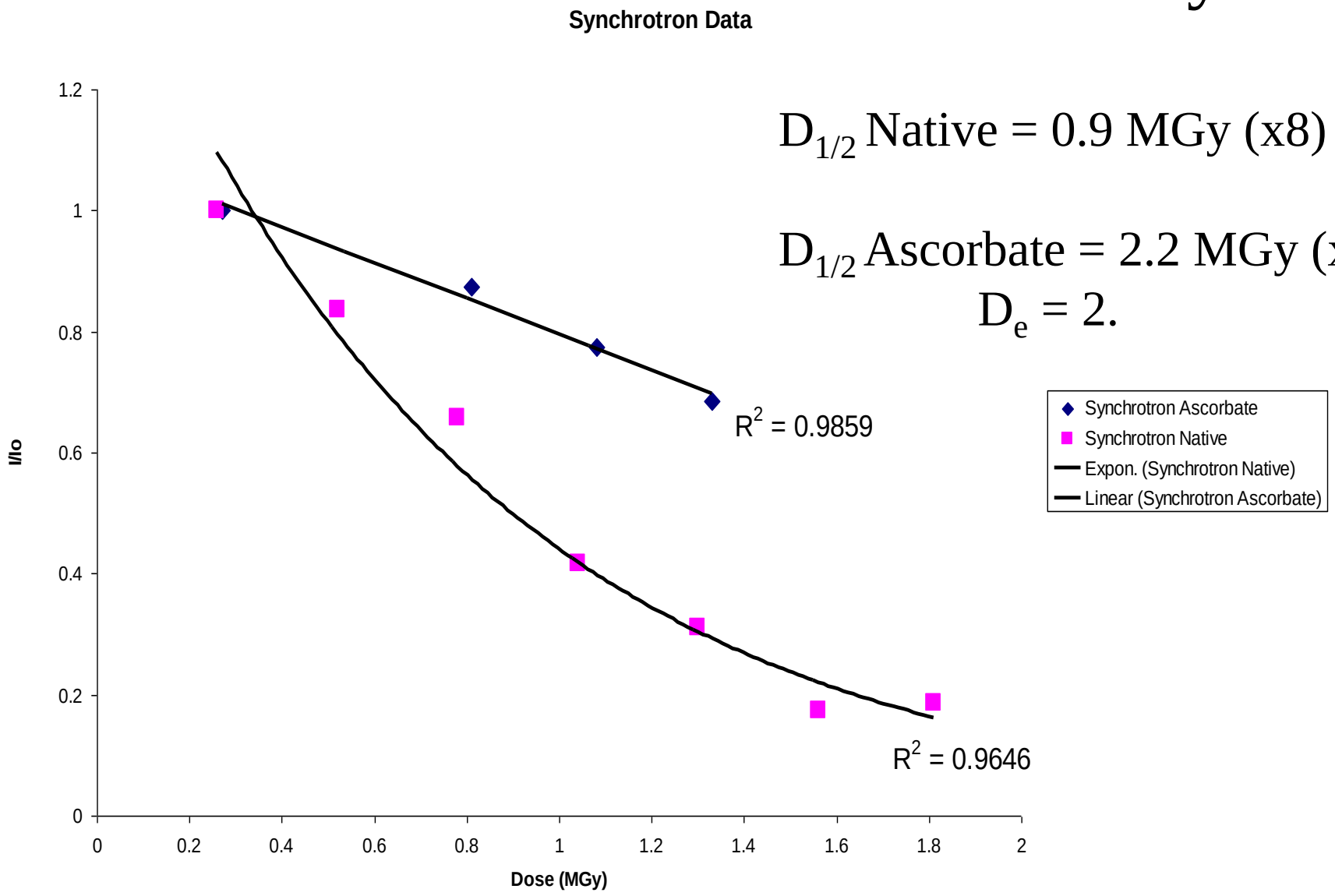
$D_{1/2}$ Ascorbate = 0.24 MGy

Native–Exponential (1st Order), Ascorbate–Linear (0th Order).

Decay of native crystals is linear at 100K – is the RT native exponential decay dominated by OH radicals?

Dose rates (Gy/Sec) = Native 1 – 6, Native 2 – 12.8
Ascorbate 1 – 6.4, Ascorbate 2 – 6.4

RT: ascorbate ESRF data: 2800 Gy/s

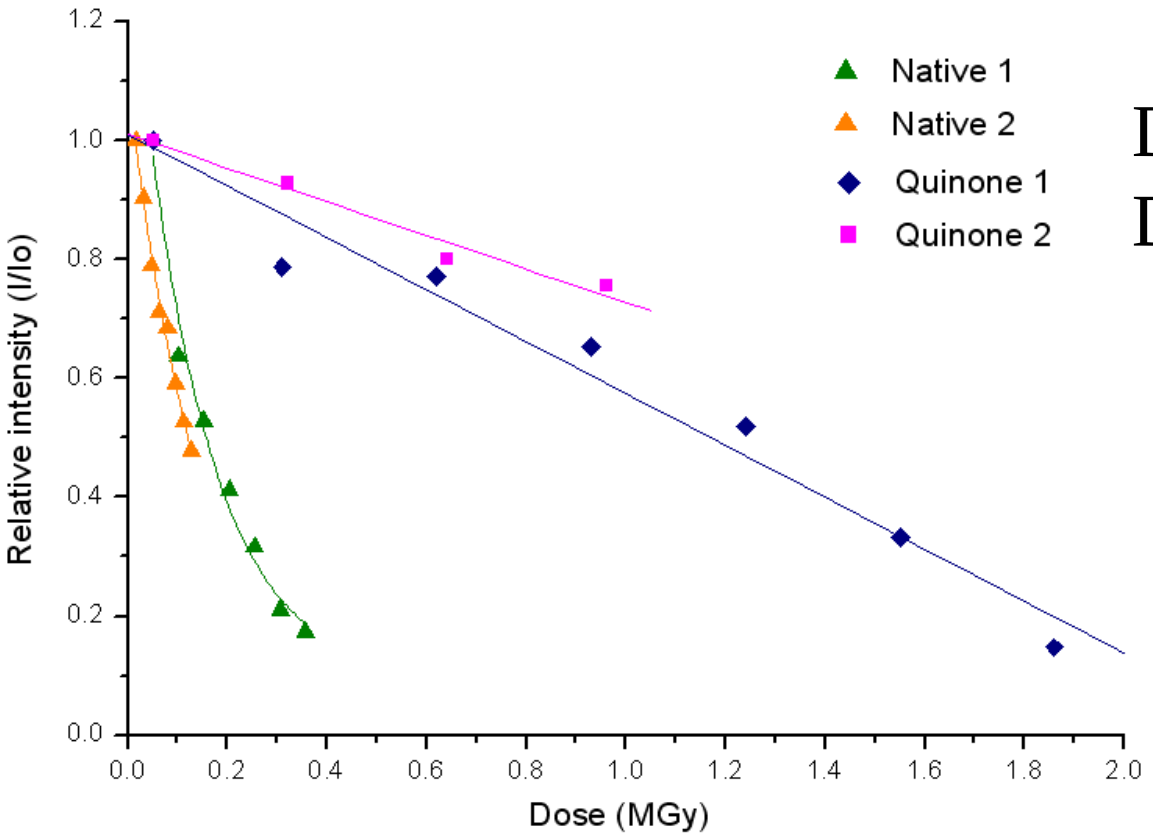


$D_{1/2}$ Native = 0.9 MGy (x8)

$D_{1/2}$ Ascorbate = 2.2 MGy (x9)

D_e = 2.

Quinone, soak, 14 days, 1M



$D_{1/2}$ Native = 0.14 MGy,
$D_{1/2}$ Quinone = 1.2 MGy

D_e = 9.

Native – Exponential (1st order), Quinone – Linear (0th).

Dose rates (Gy/Sec) = Native 1 – 6.4, Native 2 – 6.0
Quinone 1 – 5.7, Quinone 2 – 5.9

Electron density difference map analysis shows no specific damage.

Scavengers

- Effective at RT (benzo-quinone factor of 9, ascorbate factor of 2)
- Higher dose tolerance when using scavengers with higher ke-.

(Barker, A.I., Southworth-Davies, R. J., Paithankar, K.S., Carmichael, I. and Garman,E.F. *J. Synchrotron Rad.* (2009). **16**, 205–216)

- De=D1/2(scavenger)/ D1/2(native)

Sodium Ascorbate ke-= 3.0 x 10^{8} $M^{-1}s^{-1}$ k_{OH} = 8 x 10^{9} $M^{-1}s^{-1}$ De= 2

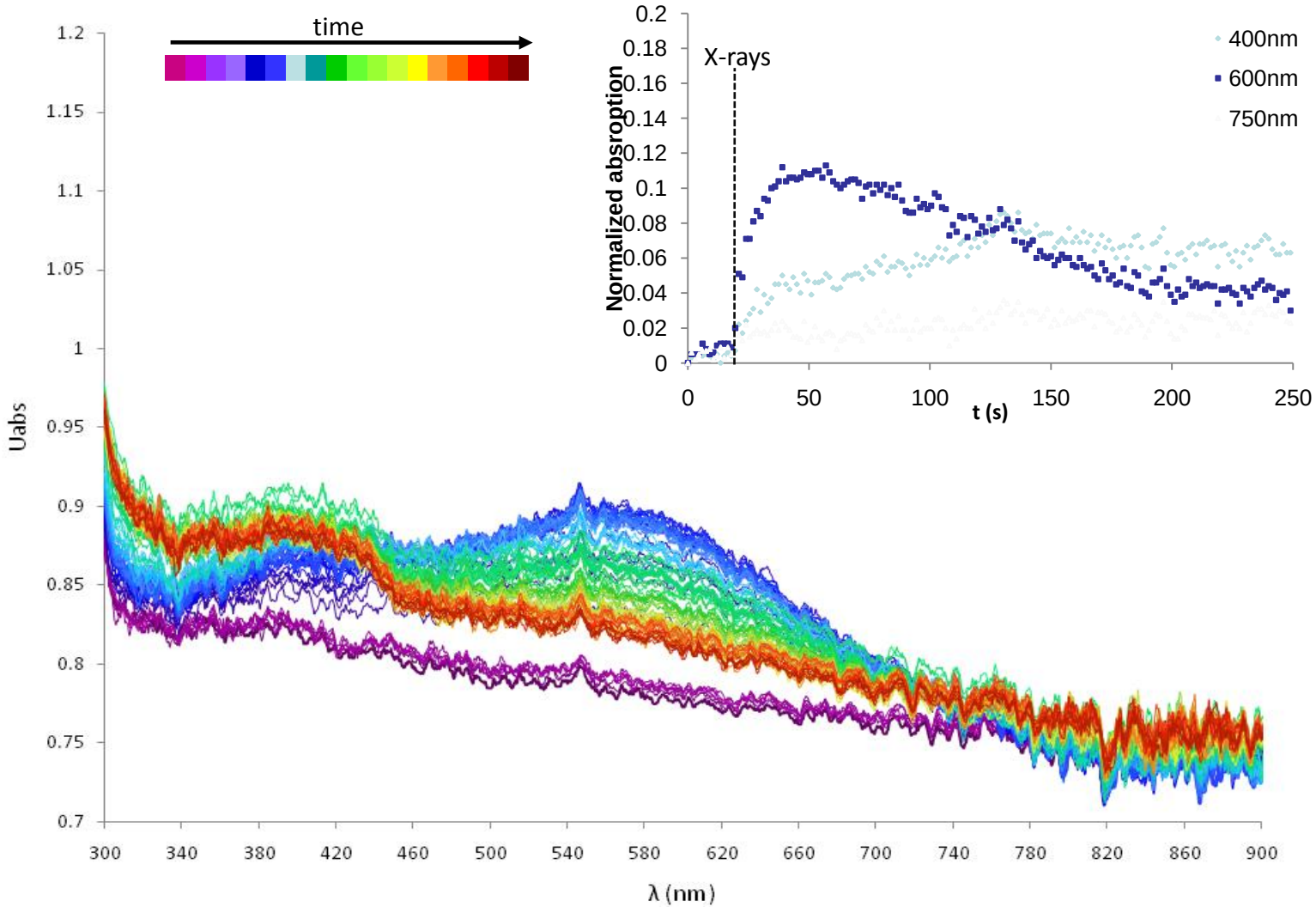
1,4-Benzoquinone ke-= 1.2 **x** 10^{10} $M^{-1}s^{-1}$ k_{OH} = 1.2 x 10^{9} $M^{-1}s^{-1}$ De= 8.9

- Not clear yet at 100K. Ascorbate is effective in surpressing specific damage.
- Is it possible to isolate e-(aq) and OH• effects?
- Try an electron scavenger:

Sodium Nitrate ke- = 9.7 x 10^{9} $M^{-1}s^{-1}$

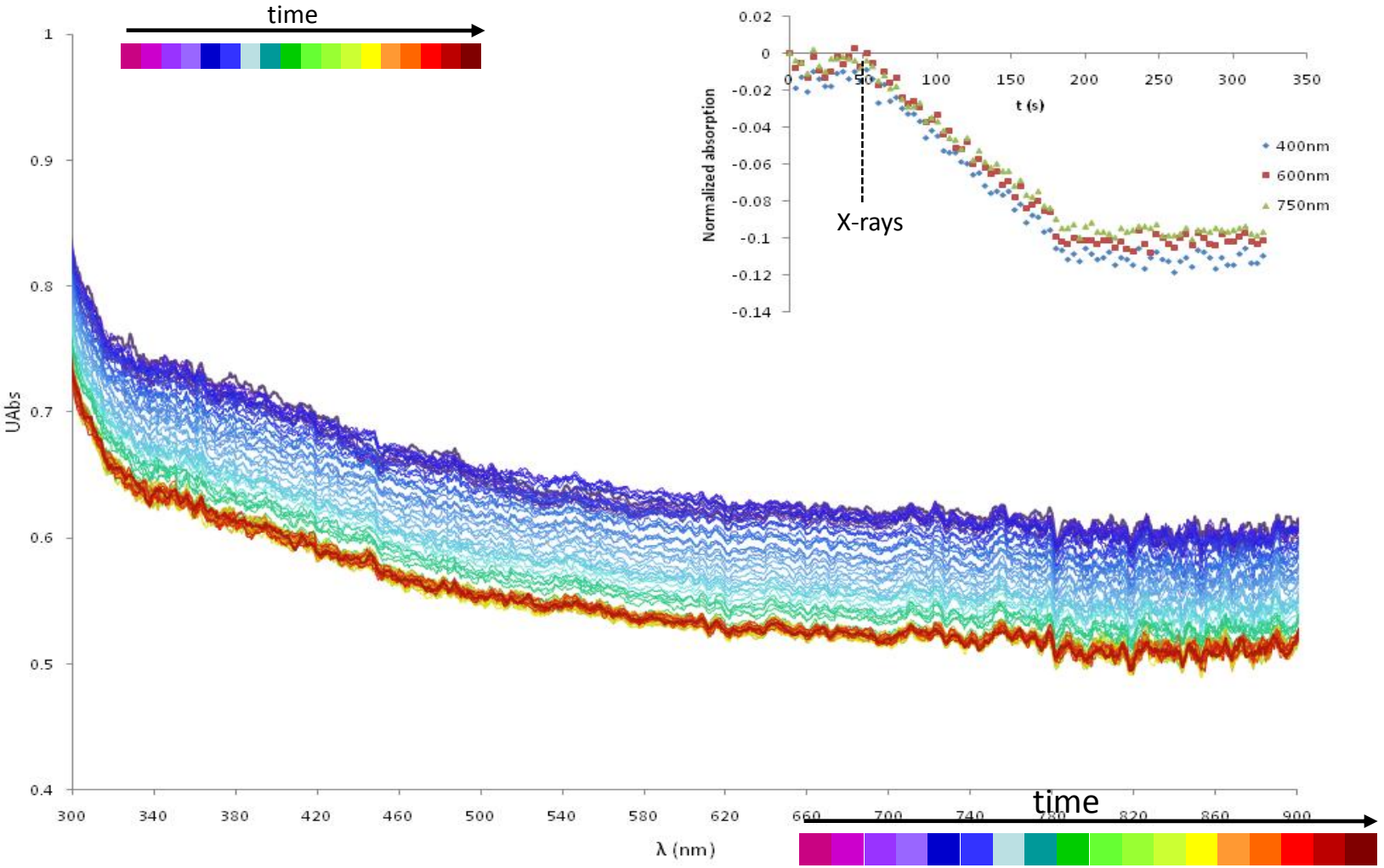
•100K: Lysozyme solution (NaAc 200mM pH 4.7, 10%w/v NaCl lysozyme 50 mg/mL, 20 % glycerol)

•e^-(aq) produced during the first fs of irradiation produced the disulfide radical anionCys· with a characteristic peak around 400 nm.



100K: Lysozyme solution + 1M sodium nitrate

$e^-_{(aq)}$ scavenged effectively.
No S-S$^\bullet$ are observed.

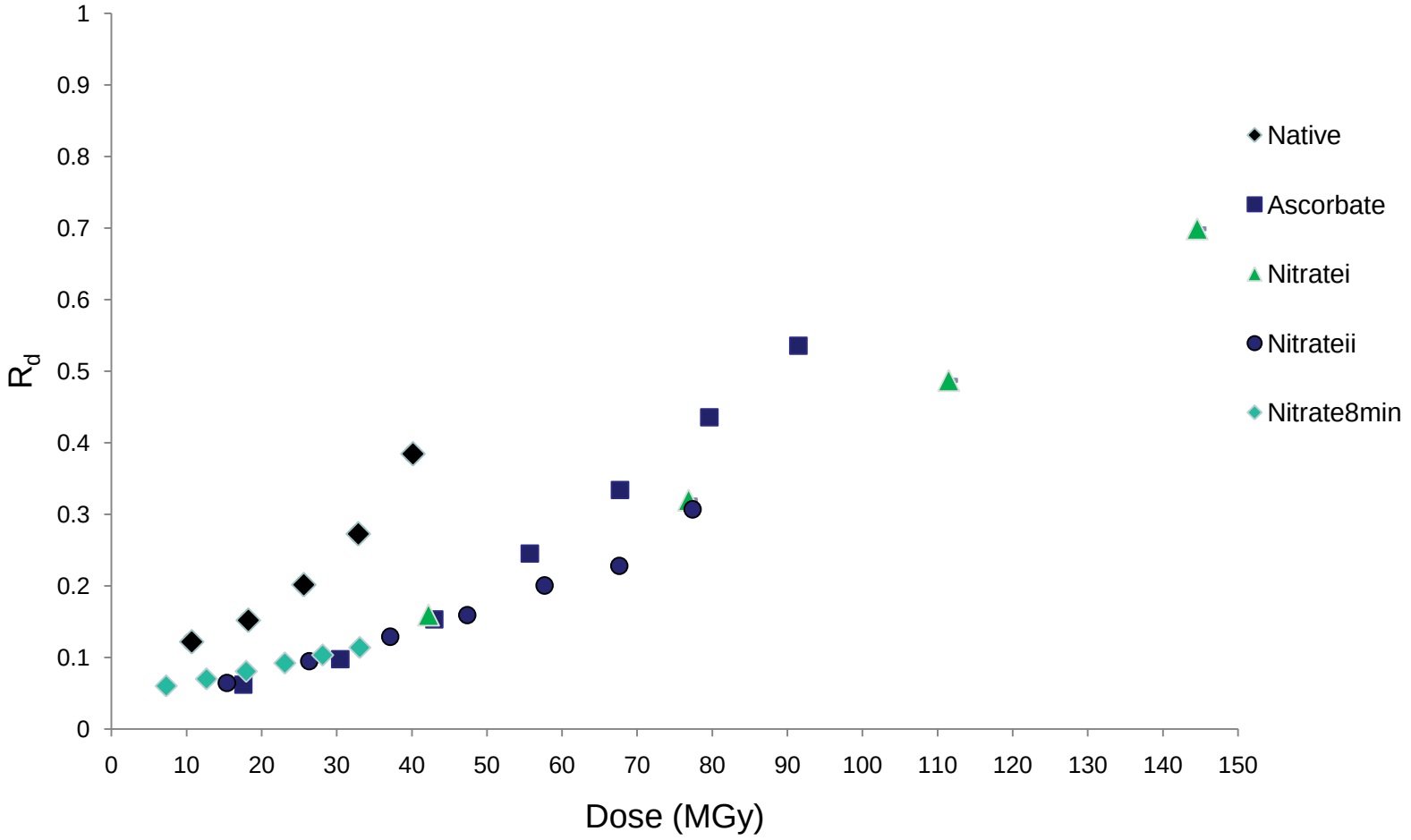


R_d

Lysozyme crystal + 1M sodium nitrate

$D_e = 3$

Protects disulfide bonds up to 70 MGy.



Radioprotectants: Conclusions

Not yet seen more than a factor of 3 in global damage at 100K, but have seen protection of amino acids so definitely worth considering for specific cases.

Have potential to make a significant difference at room temperature.

We are working on trying to understand the changes from first order to zeroth order kinetics at RT.

The Plan:

- A metric for Radiation Damage. Dose: RADDOSE.

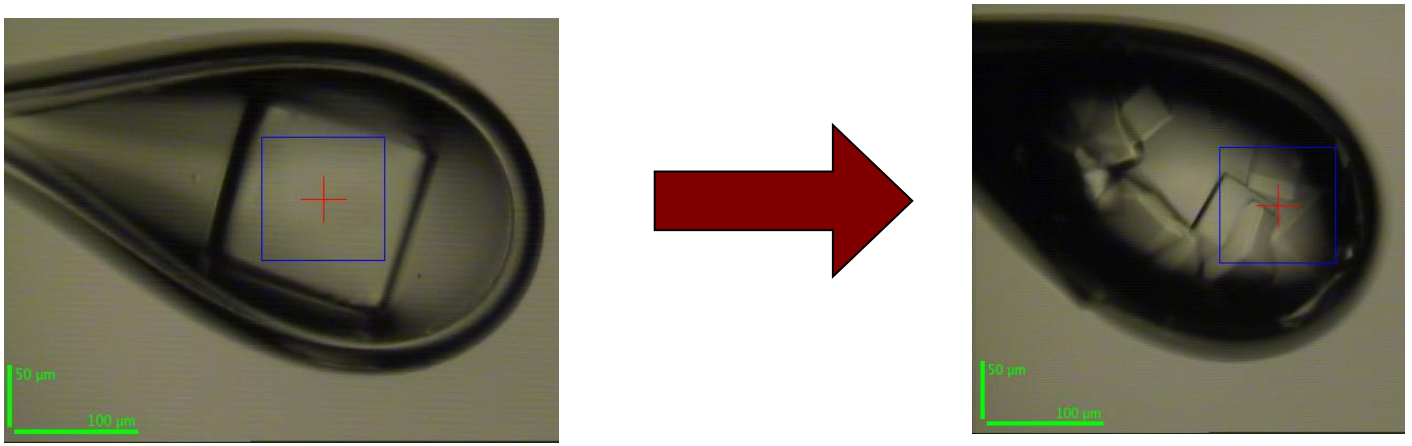
- Scavengers: RT and 100K.

- **Simultaneous multi-crystal data collection and data retrieval.**

Objectives

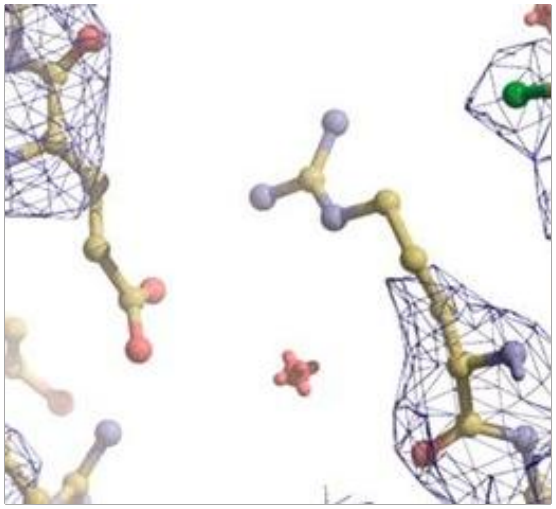
Total Cryst

- Adapt methods from material sciences to macromolecular crystallography
- Use of multiple crystals is routine in chemical crystallography
- Take multiple crystals (2, 3, ... n) in a single loop and collect data – oriented randomly
- Index the diffraction pattern and utilize the information from all the crystals
- **Test the feasibility** of the new methods for MX to combat radiation damage
- Computationally provide accurate estimate of the maximum dose limit using the program RADDOSE
- Optimise the energy incident for a given crystal size, composition



Why ? Specific structural damage

Radiation damage in crystallography even at 100 K



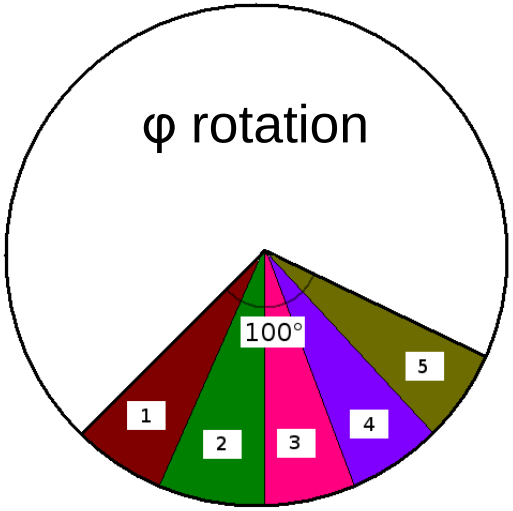
Usual: Collect single crystal dataset for 100

Proposed: collect 20 of data from 5 different crystals simultaneously and combine them

data of 20 5 crystals ~ data of 100

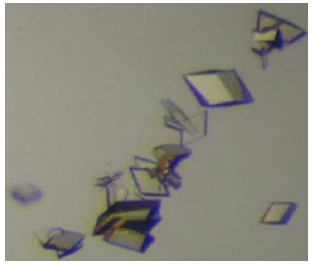
Advantages:

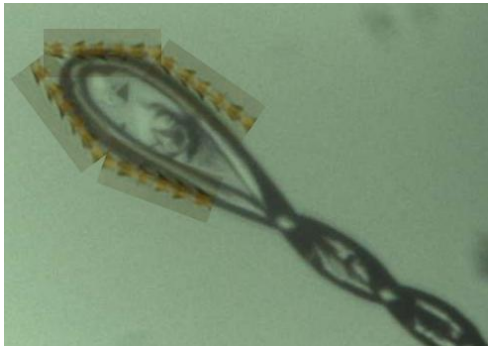
- ✓ All the 5 crystals are at the beginning of decay
- ✓ Lower absorbed dose per crystal
- ✓ Higher quality data
- ✓ Metal centres, active site preserved
- ✓ Extract correct biological information

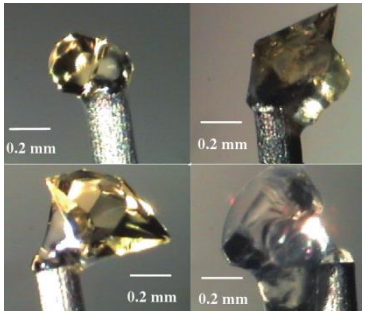


Potential applications of multiple crystal diffraction

- Micro crystals used in structure solution
- Minimize crystal handling (less mechanical damage)
- Multiple micro crystals could be mounted with Crystal catcher system
- Animal hairs attached or woven (like triple helices) on the surface of loops could be used to fish micro-crystals
- Streak seeding could be done with such loops and subsequently left in the drop (protein + precipitant) to grow crystals on their surface

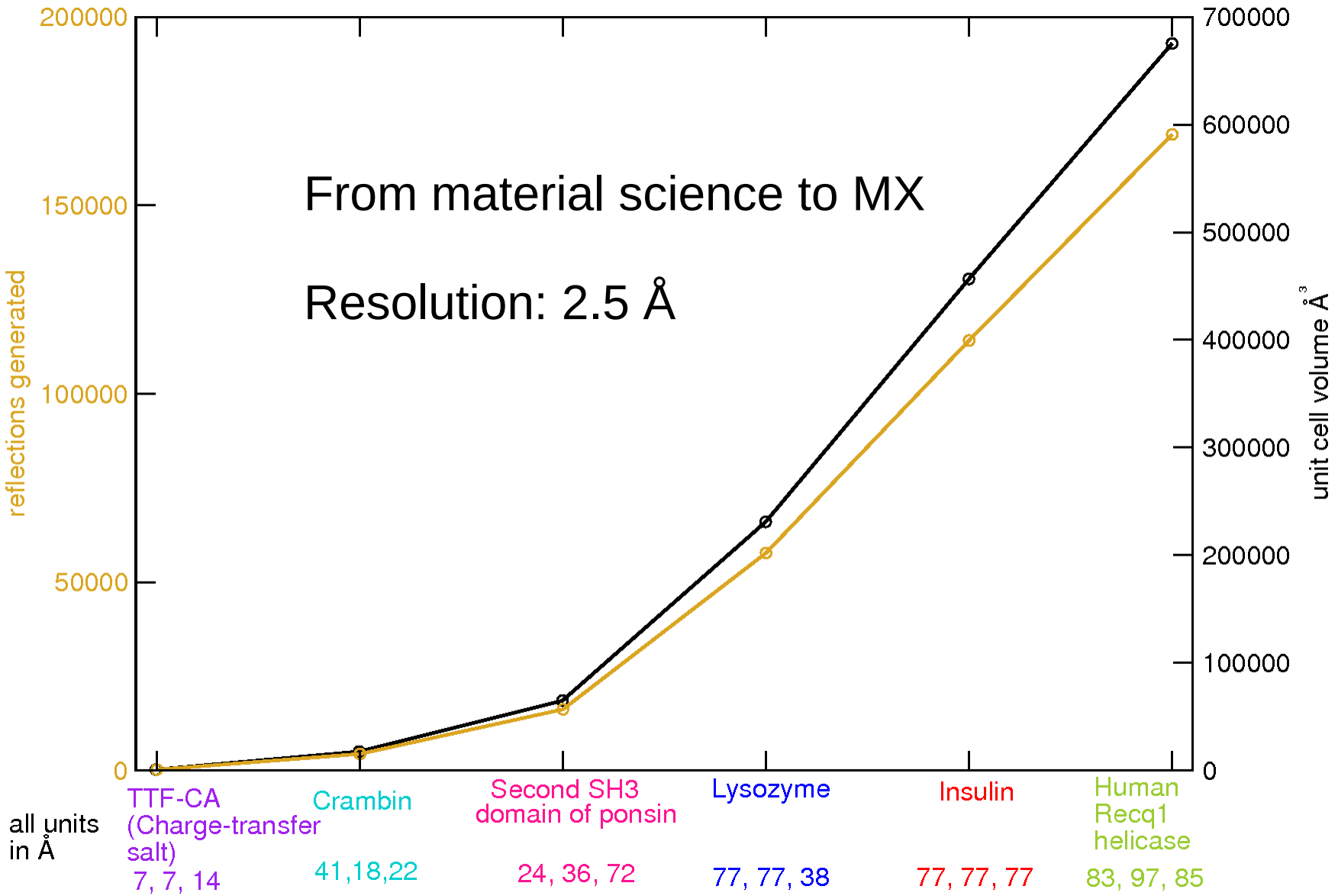






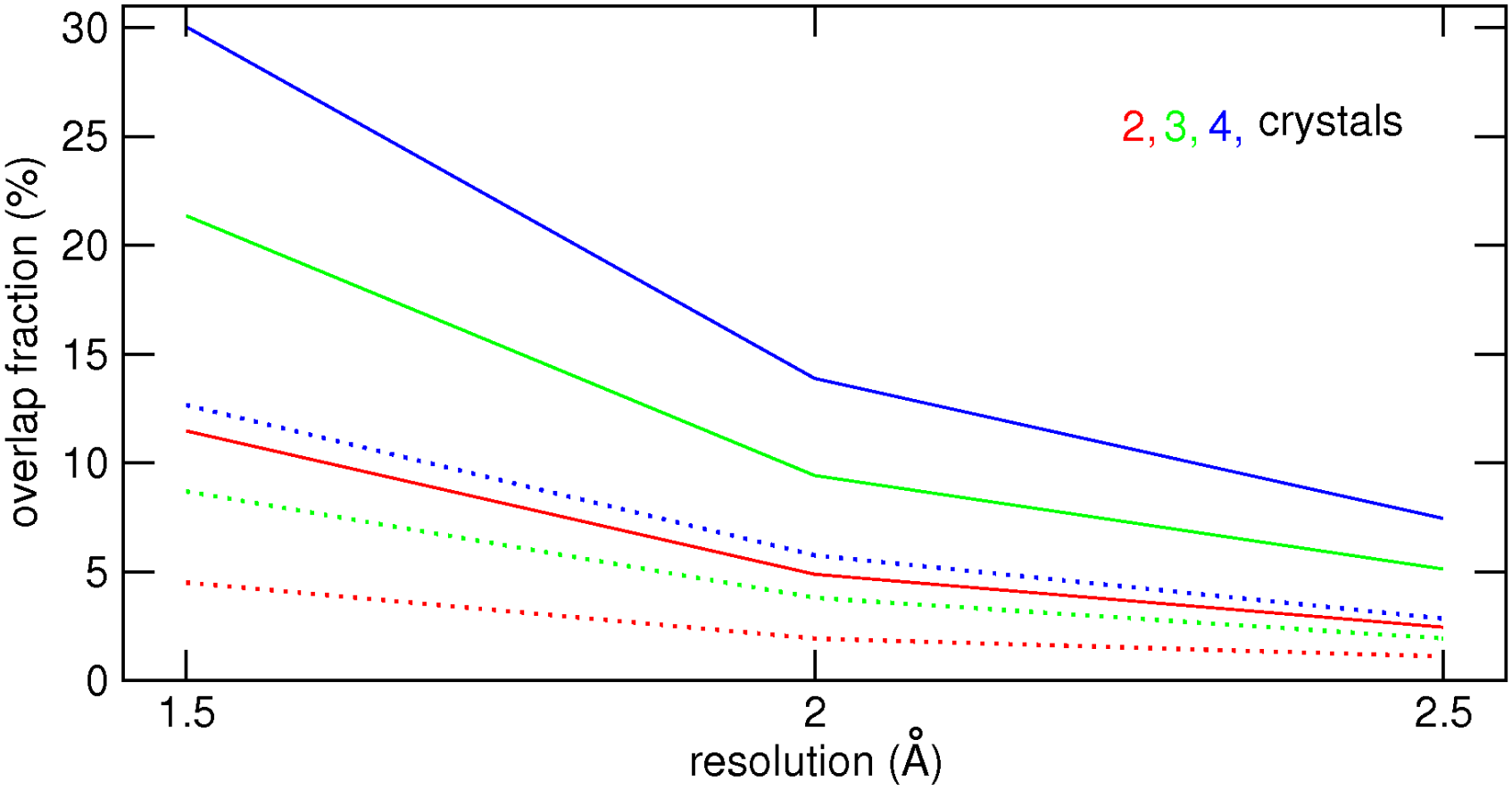
Crystal Catcher: Kitatani *et al.*, (2008) Appl. Phys. Express **1**, 370021-3

Multi-crystals: abundance of reflections leads to spot overlap



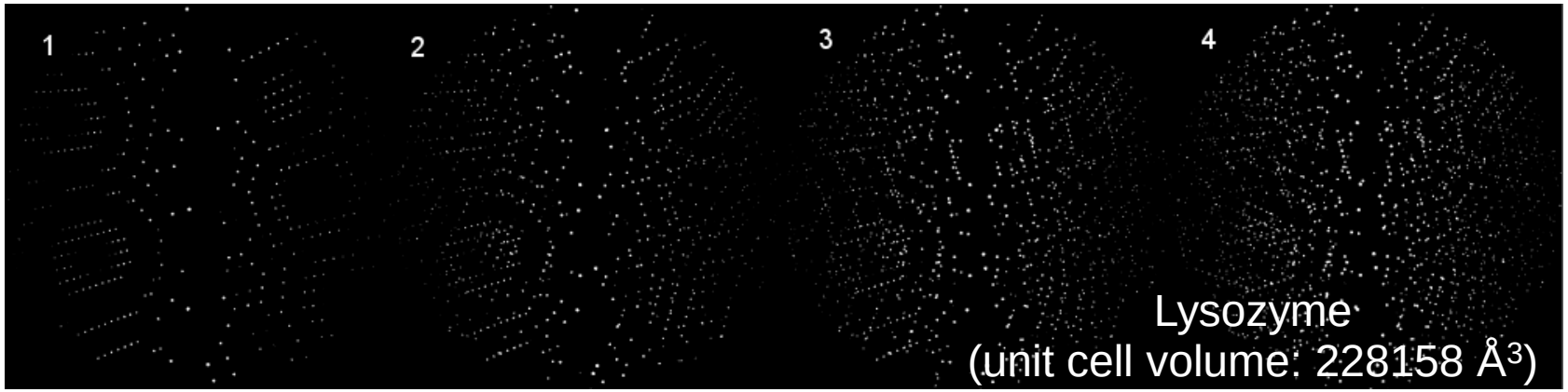
Overlap fraction with multiple crystals (mosaicity = 0.5º)

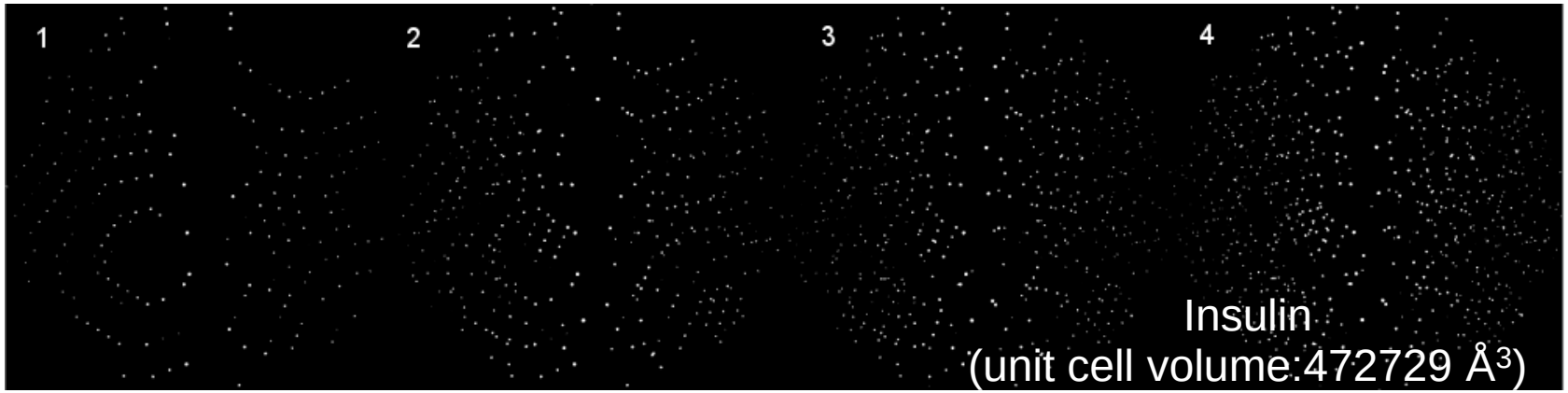
Dotted lines → Lysozyme
Solid lines → Insulin



Simulations of diffraction patterns

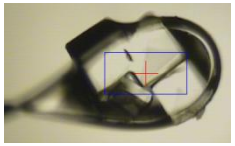
Diffraction patterns with 1, 2, 3 and 4 crystals of lysozyme (top) and insulin (bottom), $\Delta\varphi = 0.5^{o}$





Overview of the methodology

Total Cryst



RADDOSE

Data acquisition

Harvest reflections

hkl, I, σI

Integrated intensities

Indexing

3D – structure

MX tools

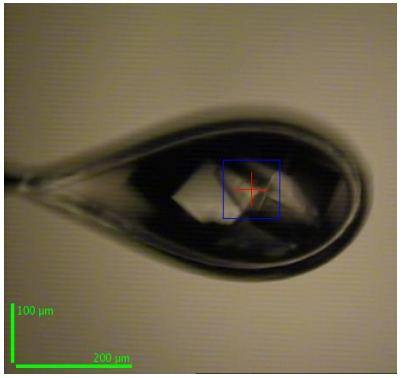
Crystal Orientation

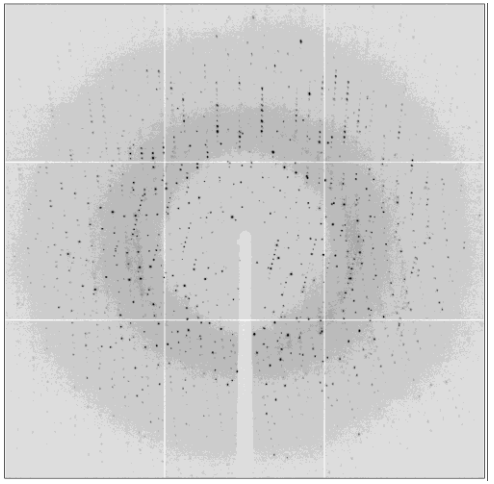
Spot prediction

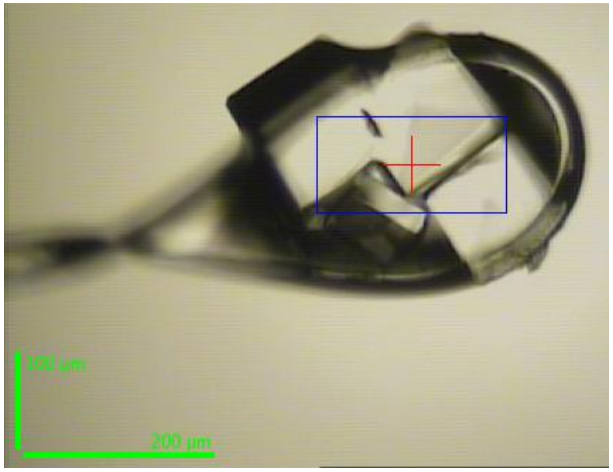
Material scientists

Experiments: Multiple crystals in X-ray beam

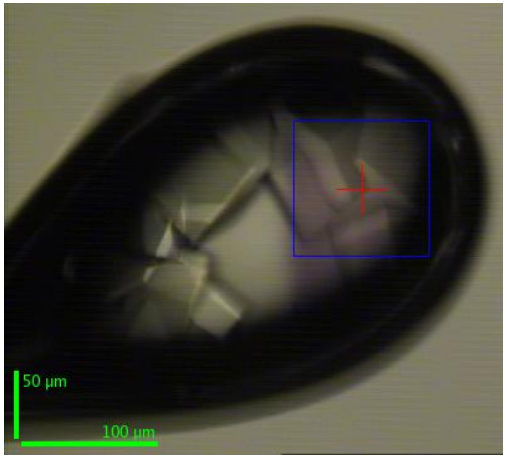
Data of crystals from lysozyme and insulin collected at ESRF (ID 14-4)



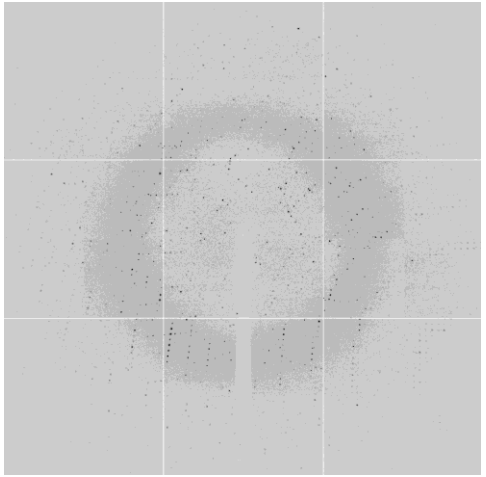




Insulin

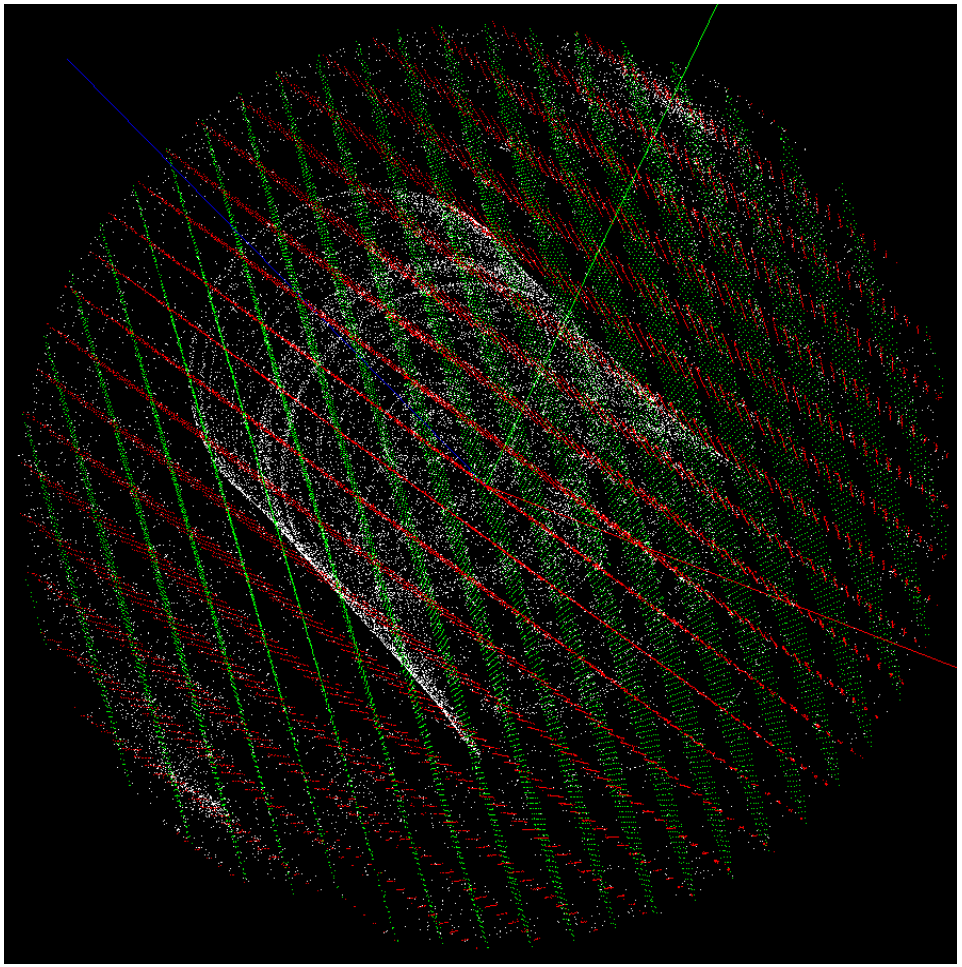


Lysozyme



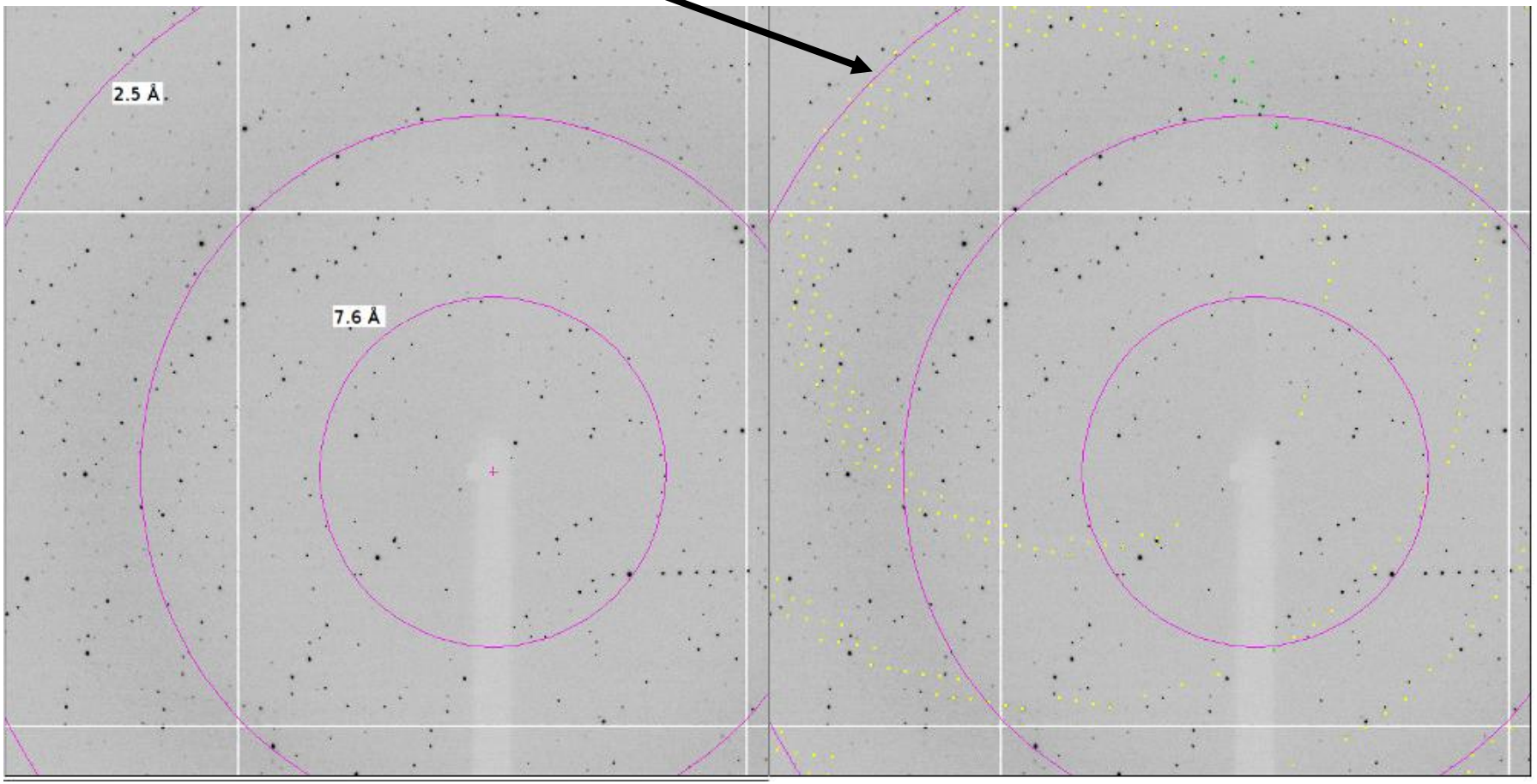
Data reduction in practice using materials science software

- Search for peaks above a certain threshold and construct an array of peaks
- Indexing with pattern recognition algorithm - Grainspotter
- OUTPUT: orientation of each crystal in the ensemble
- The orientation of each crystal lattice is provided to MOSFLM by means of the U (orientation) matrix and data integrated
- Red and green indicate the two different lattices from two crystal lysozyme dataset

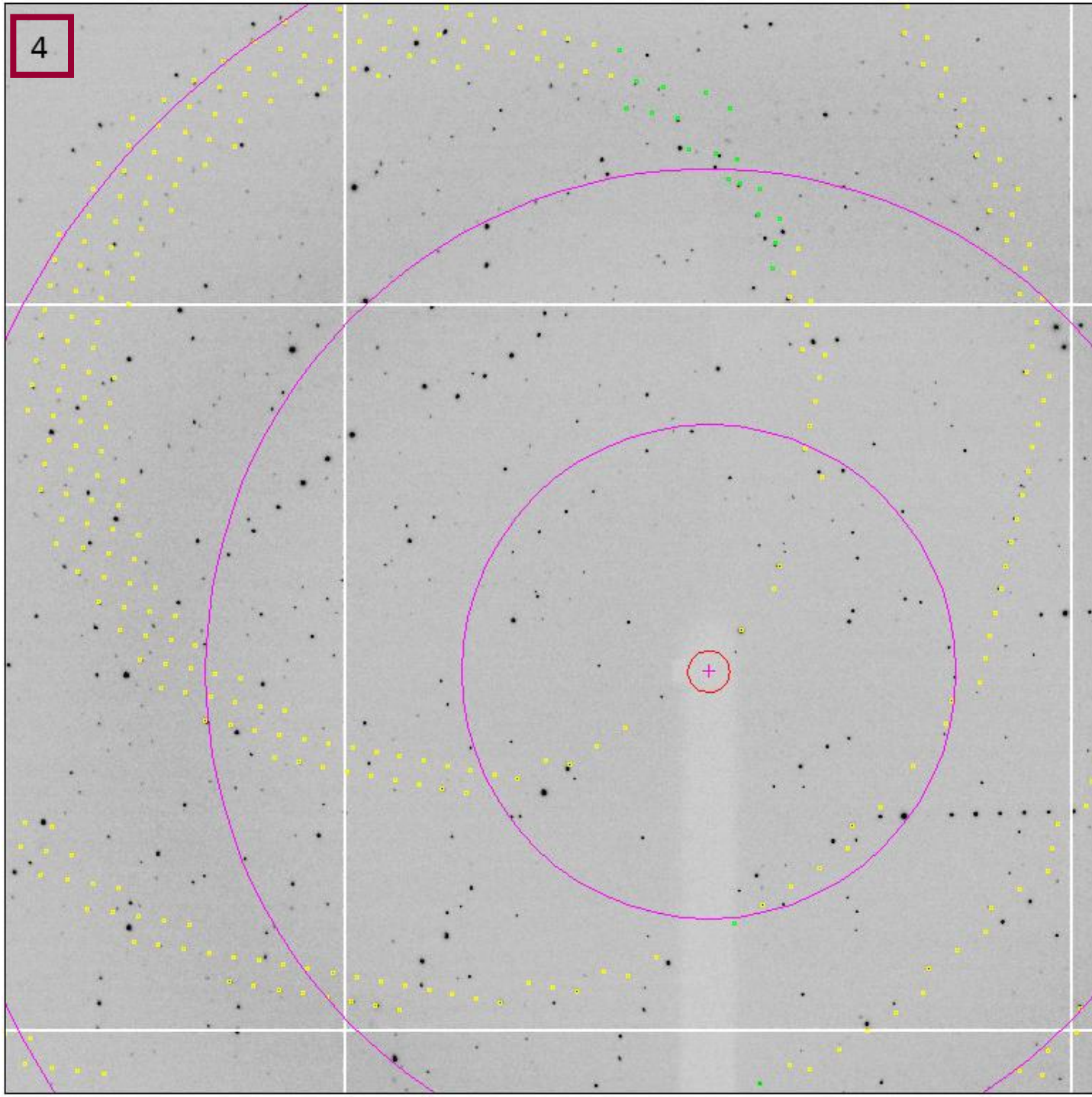


www.totalcryst.dk (all programs released as open source: www.sf.net

A single lattice from a four crystal Lysozyme dataset



Animation illustrates the four lattices identified uniquely from a dataset obtained from a multiple crystal data collection with four crystals in the beam



Summary – *TotalCryst*

Simulations show spot overlap is not huge problem; due to random overlap and not systematic overlap

Data were collected with multiple single crystals in a single loop

Possible to index unknown lattice but collecting few exposures with only one crystal or select an exposure with one strong lattice

Extraction of data from up to 7 crystals achieved

The combination of data from multiple crystals compensates for the loss of redundancy owing to rejected spots

Need to extend the experiments to microcrystals.

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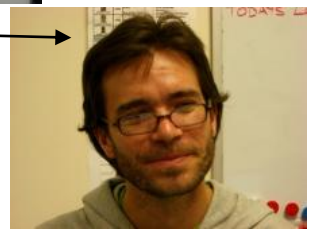

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Total Cryst
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